SURVIVAL PATHWAYS IN BREAST CANCER CELLS DURING GLUCOSE DEPRIVATION

by

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ABSTRACT

Cancer cells often encounter an environment of metabolic stress, including nutrient deprivation, low oxygen levels, or both. Cells respond to these and other types of stress, in part by using the physiological mechanism of autophagy. Autophagy is a type of "self-eating", where organelles and macromolecules are recycled for use by cells for simple molecules and energy. While autophagy is recognized to be critical for survival of cancer cells in many situations, the mechanisms contributing to cell survival during glucose deprivation are incompletely understood. In order to enhance the treatment of cancer, the potential survival and signaling pathways important in the microenvironment of a tumor need to be elucidated.

The goal of this thesis was to understand the role of autophagy and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) as mechanisms of cell survival in both glucose deprivation and hypoxia. Likewise, the role of glucose deprivation and autophagy on human epidermal growth factor receptor 2 (HER2) and estrogen receptor (ER) in breast cancer and response to targeted drug therapies was studied. Using pharmacological and genetic inhibition of both key autophagy proteins and Nrf2, we show that autophagy and Nrf2 signaling are important for the survival of cells exposed to glucose deprivation. However, these two survival pathways appear to be activated independently of one another. Nrf2 was also shown to affect survival in hypoxia. In unrelated studies to Nrf2, both estrogen and human epidermal growth factor receptors were discovered to be downregulated in response to glucose deprivation perhaps through a translational mechanism. Despite this downregulation of ER in MCF7 and T47D cells,

4-hydroxytamoxifen was still efficacious. All of these studies were conducted to obtain a better understanding of how the microenvironment of a tumor can affect the survival of cells and their response to therapies in order to target these survival pathways and improve cancer treatment.

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Chapter 1: Introduction

Cancer cells often encounter an environment of metabolic stress, including nutrient deprivation, low oxygen levels, or both. Cells respond to these and other types of stress, in part by using the physiological mechanism of autophagy. Autophagy is a type of "self-eating", where organelles and macromolecules are recycled for use by cells for simple molecules and energy. While autophagy is recognized to be critical for survival of cancer cells in many situations, the mechanisms contributing to cell survival during glucose deprivation are incompletely understood. In order to enhance the treatment of cancer, the potential survival and signaling pathways important in the microenvironment of a tumor need to be elucidated.

This thesis will describe experimental work investigating mechanisms of cell survival during glucose deprivation, particularly focusing on the role of autophagy and of Nuclear Factor erythroid derived –like 2 (Nrf2) pathway. In addition to these pathways, glucose deprivation in relation to hypoxia and important signaling pathways in breast cancer will be discussed.

Chapter 2 Background

2.1 Role of autophagy in normal physiology

Autophagy (and more specifically macroautophagy) is a term that literally means "self-eating". Autophagy is a physiologic process where a double-membraned autophagosome forms around organelles or macromolecules in the cytoplasm and fuses with a lysosome, leading to lysosomal contents being degraded into small molecules that can be used by the cell to sustain itself (1). Autophagy is critical for removing damaged organelles and macromolecules, as well as for generating energy and new substrates for biosynthesis when the cellular environment is insufficient in providing such substrates. Most likely because of the importance of autophagy in recycling defective organelles and preventing accumulation of aggregated proteins, this process occurs in normal cells at a basal level acting as a housekeeping function, and cancer cells appear to have a variably increased dependence on autophagy, possibly due to cancer cells having a greater level of metabolic stress (1-3). This importance of autophagy during nutrient deprivation was demonstrated by experiments showing that neonatal mice deficient in ATG7 or ATG5, key proteins in the pathway, die one day after birth, presumably due to lack of amino acids that autophagy would normally have maintained during the postpartum period before pups are able to obtain nutrients through nursing (4, 5). Moreover, this need for autophagy to provide nutrients during a time of inadequate supply is seen in many other circumstances.

In cells deficient in ability to undergo apoptosis, autophagy is induced in order to maintain nutrients and ATP levels when growth factors are scarce. Inhibition of

autophagy leads to death of these cells unless growth factors are reintroduced, in which case proliferation can be resumed (6). For example, in cardiomyocytes it was found that during glucose deprivation, hexokinase II, an enzyme in the first step of glycolysis, binds to and inhibits mTORC1, thus inducing autophagy (7). Breast cells undergoing extracellular matrix detachment also induce autophagy in order to survive, and without autophagy, these cells undergo apoptosis (8). Autophagy is thought to be important in all cells of the body for maintaining homeostasis as well as for allowing survival during metabolic stress.

2.2 Role of autophagy in cancer/ Paradox of tumor suppression vs tumor cell survival

Over 80 years ago, Otto Warburg described a phenomenon whereby cancer cells metabolize glucose through glycolysis to pyruvate and lactate, foregoing the oxidation of pyruvate in mitochondria, even in the presence of adequate oxygen for the process of oxidative phosphorylation. This process of anaerobic glycolysis is less efficient for generating ATP than oxidative phosphorylation. With the high demand for glucose, cancer cells commonly have correspondingly increased levels of glucose transporters on the cell membrane (9), but any deficiency in the availability of exogenous glucose requires the recycling of endogenous, sources of energy, a demand that can be met by autophagy. As expected, autophagy appears to be particularly prevalent in tumor regions distant from blood vessels, which are presumed to have metabolic stress (10). Tumor cell autophagy, which commonly results from inadequate blood supply, parallels the responses seen in normal cells encountering similar stresses, with a lysosomal

degradation pathway activated by starvation, hypoxia, or growth factor deprivation (2). In the acidic environment, found in tumors due to the Warburg effect, lactate and other acidic metabolites cause upregulation of phosphorylated AMPK leading to decreased mTOR signaling and activation of autophagy. This process has been shown to protect melanoma cells (11). Autophagy can also be a mechanism for cell death, and, prolonged hypoxia in a variety of apoptosis-competent cell lines was found to induce autophagic cell death (12).

The exact role of autophagy in cancer appears to be complex, depending on factors that include the context, type and stage of the tumor. Some studies have suggested that autophagy has a suppressive role during tumor initiation. Evidence to support this role include findings that Beclin 1, a protein involved in induction of autophagy, is a haploinsufficient tumor suppressor in breast cancer (13-16). In clinical tissue samples of breast cancer, Liang et al. observed that all adjacent normal tissues stained for Beclin 1, but over half of the tumors showed a decrease in Beclin 1 expression (14). Interestingly, induced expression of Beclin 1 in MCF7 breast cancer cells results in a less malignant phenotype and slower proliferation (14). Clearly the absence of important autophagic proteins results in defective autophagy with these cells having more DNA double strand breaks, gene amplifications, and damaged mitochondria, along with p62 accumulation which cause an increase in reactive oxygen species (ROS) (15, 17). When autophagy is available to limit levels of ROS, suppression of tumorigenesis in immortalized baby mouse kidney cells expressing activated RAS occurs, and by limiting necrosis in apoptosis-deficient cells, inflammatory cells that would infiltrate the tumor and promote tumor growth are quelled (15). Thus, in some situations, autophagy appears to prevent

tumorigenesis through clearance of damaged organelles, maintaining energy homeostasis, and limiting genome damage (2).

In other settings, however, autophagy appears to be important for cancer cell survival. For example, during hypoxia the HIF1α transcription factor induces BNIP3L, causing disruption of the Beclin1-Bcl2 complex and release of Beclin 1, thus inducing autophagy (2, 12). In this setting, autophagy provides nutrients to the tumor facing an increased metabolic demand, and is thus a survival response. In tumors, chemotherapeutic drugs also contribute to metabolic stress, and activation of autophagy in this setting can cause resistance to the chemotherapy. Supporting this concept are findings that inhibiting autophagy during chemotherapy may limit resistance to the drugs (18). Autophagy is also believed to be involved in dormancy during metabolic stress, and when nutrients become plentiful again, the cell can resume proliferation (2). Similarly, autophagy is thought to be important for resistance to anoikis, and thus may provide a mechanism for survival during metastasis (8, 15). As can be seen by the examples above, there is evidence for both a tumor-promoting and tumor-preventing role of autophagy.

2.3 Molecular mechanisms of autophagy

Autophagy is regulated by over 32 autophagy-specific proteins and involves multiple energy and nutrient sensing pathways, including AMPK, PI3K, HIF1, and mTOR. The mTOR pathway involves mTORC1, which is sensitive to the availability of nutrients. mTORC1 interacts with Raptor to directly phosphorylate p70S6K and 4E-BP1, which in turn regulate protein synthesis. Another pathway used by the cell to monitor its metabolic status is AMPK. AMPK is considered to be an energy sensor of the cell,

monitoring the AMP: ATP ratio and phosphorylating the protein, Raptor, when activated by relatively increased levels of AMP that occur in situations of low nutrients or metabolic stress. Phosphorylated Raptor, in turn, inhibits mTORC1 (or directly phosphorylates ULK1 and ULK2), blocking anabolic processes and initiating autophagy (18-21). Moruno, et al. interestingly found that addition of glucose to cells after a period of starvation can also induce autophagy, although this induction occurs through the MAPK pathway and not AMPK (21). MAPK family member, JNK, phosphorylates Bcl2, interrupting its complex with Beclin1 and inducing autophagy (18, 22). Notably, Bcl2 can be both anti-apoptotic and anti- autophagic, with inhibition of autophagy resulting from its binding to Beclin1's BH3 domain (13). Ras mutations in cancer can also activate the ERK pathway, which inhibits mTORC1, or p38 can trigger AMPK, both of which lead to induction of autophagy (18). AMPK is apparently important in MEK/ERK regulation of autophagy, as it was shown that defective AMPK could no longer phosphorylate and activate MEK to upregulate autophagy. Also when MEK and ERK are inhibited Beclin 1 expression decreases and autophagy is downregulated (23). Additionally, the PI3K pathway can suppress autophagy through activating AKT which inhibits the TSC1/TSC2 complex, de-repressing mTORC1 (18).

Under nutrient rich conditions, mTOR, Unc-51-like Kinase 1 (ULK1), Unc-51-like Kinase 2 (ULK2), ATG13 and focal adhesion kinase family-interacting protein of 200kD (FIP200) form a complex, wherein mTORC1 binds to ULK1 through Raptor, and through subsequent phosphorylation of the ULKs prevents induction of autophagy (3, 24). During nutrient starvation, mTORC1 dissociates from ULKs resulting in their dephosphorylation and activation to then phosphorylate ATG13 and FIP200 (3, 24, 25).

Induction, the first step of autophagy occurs when starvation or treatment with an mTOR inhibitor, such as rapamycin, causes ULK1 and ULK2 to form a complex with FIP200 and ATG13 (24, 25).

The second step of autophagy, nucleation, begins when Beclin 1 recruits vps34 to a complex that also consists of p150 (3, 26, 27). Next, vesicle elongation occurs when ATG12 and ATG5 are covalently linked by ATG7 and ATG10, E1 and E2-like proteins respectively (3, 24, 25, 27). The ATG12-ATG5 complex then binds with ATG16L to form the preautophagosomal structure (3, 24, 25, 27). LC3 is cleaved by ATG4 at its carboxyl terminal to generate LC31, which is conjugated to a phosphatidylethanolamine by ATG7 and ATG3 to form LC3II (3, 24, 25, 27). The ATG12 complex assists in the conversion of soluble LC3I to membrane bound LC3II, the form found on mature autophagosomes, until the autophagosome fuses with a lysosome and degrades releasing its contents into the cytoplasm where they can be used for metabolism and synthesis of new proteins (3, 24, 25, 27). Nucleosides generated can be used to make ATP or converted to glucose using the pentose phosphate pathway (PPP). Amino acids can be shuttled into various points of the tricarboxylic acid cycle (TCA) (3).

Autophagy is for the most part non-selective, but there is a molecular mechanism for cargo recognition (16, 25). Cargo selectivity involves ubiquitinated, aggregated proteins that need to be cleared from the cell. P62/SQSTM1 binds to these poly-ubiquitinated substrates via an ubiquitin associated domain, linking the cargo to microtubule associated protein light chain 3 (LC3) on the outside of the autophagosome (25). Out of all the stages, the formation of the autophagosome and the blocking of autophagic flux are the processes that are often measured to monitor autophagy.

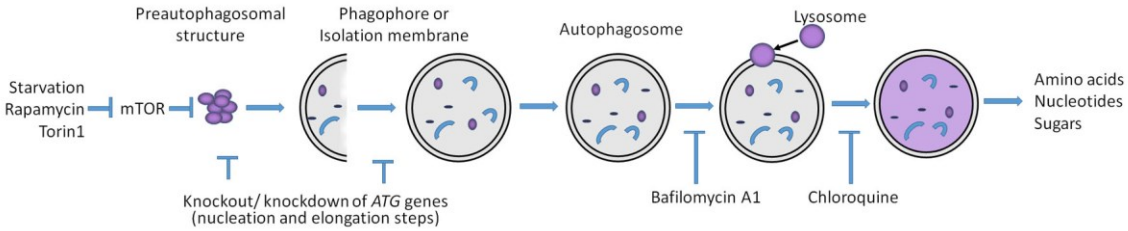

Figure 2.1 Autophagic Process, adapted from "Methods in Mammalian Autophagy" (28)

2.4 Measuring molecular events in autophagy

There are several methods used to measure autophagy, including both steady-state methods and flux measurements. Steady-state methods that detect induction of autophagy include electron microscopy, immunoblotting to measure upregulation of LC3II, and fluorescence microscopy to monitor autophagosome formation. While measuring induction of autophagy is important, measurements of autophagy flux are even more informative for determining whether the process is being completed. Methods to assess completion of autophagy include measuring the turnover of the LC3II protein, monitoring GFP-LC3, measurements of the p62 protein, and microscopy to monitor autophagosomes. Autophagy can also be probed by disrupting the process with xenobiotics, including acidotropic dyes and inhibitors of autophagy (29). However, it is important to be aware of limitations to use of chemical inhibitors, since these agents can have non-specific effects; therefore, their use should be supplemented with genetic modification or blockage of the pathway (30).

Molecular assays are used for more quantitative assessment of the process. During autophagy, LC3I gets conjugated with phosphatidylethanolamine to form LC3II. LC3II migrates faster on a gel due to its greater hydrophobicity and thus the two bands of LC3 can be separated from each other by electrophoresis. When measuring these forms of LC3, one should normalize to a loading control rather than LC3I, due to the instability of LC3I and the different affinity some antibodies have for LC3I. To assess flux, comparable samples can be treated with an autophagic inhibitor followed by measurements of LC3II or p62. P62, a protein that brings cargo to the autophagosome, is used to indicate a block in autophagy and reduced turnover of ubiquitinated proteins (29, 30). Increased p62 or LC3II observed in a sample treated with an autophagy inhibitor compared to a non- inhibitor treated sample is indicative of the autophagic process going to completion and degrading the contents of the autophagosome, as opposed to being blocked at an upstream step. There are many ways to measure autophagy and multiple methods should be used to ensure robust data.

2.5 Evidence for a potential role of Nrf2 in cell survival during autophagy

As discussed above, autophagy can be a mechanism of cell death as well as a mechanism for cell survival, particularly during cell stress. One molecular pathway that we have found to be important for cell survival during glucose deprivation and potentially during autophagy is the Nrf2 (nuclear factor erythroid derived –like 2) pathway. Autophagy and the Nrf2 pathway have previously been shown to be connected through the protein p62. P62 facilitates degradation of ubiquitinated proteins through autophagy using an ubiquitin binding domain on the c-terminal and LC3 interacting

region (31). mTOR, which inhibits autophagy, directly phosphorylates p62 on S351, causing a strong affinity for Keap1 (32) and thus increased Nrf2 activation.

Accordingly, Nrf2 target gene expression has been shown to be suppressed during treatment with rapamycin, an mTOR inhibitor that also decreases p62 phosphorylation (32). Experiments involving ATG7 deficient cells and mice have found that autophagy leads to increased accumulation of p62, co-localization of p62 and Keap1 and induction of Nrf2 (32-34). Autophagy is important for tumor growth in these mice, and while ATG7 deficient mice show increased early tumor growth, growth of tumors later slows down due to poor mitochondrial function and metabolic deficiencies in tumor cells. Mice deficient in Nrf2 also show increased early tumor growth but increased survival of mice due to slow tumor growth later. Initially, elevated ROS production is most likely beneficial for early tumor growth, but ROS appears to not be involved in the slower growth later in tumor development and reduced tumor burden seen in ATG7- or Nrf2-deficient mice, compared to controls (34) Interestingly, arsenic upregulates the quantity of autophagosomes through inhibition of fusion with the lysosome and acidification. Arsenic, like the ATG7 knockout mice, causes p62 accumulation leading to sequestration of Keap1 and activation of Nrf2. In this case, the activation of Nrf2 was due to p62 accumulation rather than increased ROS.

The role of reactive oxygen species (ROS) in autophagy and effects of ROS on tumor growth have been studied by other investigators. It was shown by Wang, et al. that treatment of PC12 cells with melamine results in increased ROS, but the autophagic response helps to inhibit the excessive production of ROS (35). Autophagy is thought to be protective by preventing accumulation of ROS that may damage proteins or DNA

(36), and cells deficient in the ATG7 gene have higher levels of ROS (37). Autophagy might be particularly important for the regulation of ROS in cancers with RAS mutations, where autophagy is needed by the cell to tolerate the extra mitochondrial and metabolic stress (36).

ROS produced from mitochondria during oxidative phosphorylation can also indirectly regulate autophagy induction through AMPK (36) , and blocking ROS can result in decreased autophagy associated with decreased AMPK activation (38). Another clue for how ROS can induce autophagy comes from experiments showing that hydrogen peroxide can directly oxidize ATG4, leading to increased formation of LC3II. Other work has found that with knock down of Nrf2, autophagy increases in order to deal with the reactive oxygen species, however, when Nrf2 is present at high levels, less autophagy is needed because of the ability of Nrf2 to defend the cell from ROS on its own (39).

Sestrins are another group of autophagy-related proteins involved in the defense against ROS. The Nrf2 pathway also mediates the defenses of sestrins, and increases in either SESN1 or SESN2 reduce Keap1 protein and upregulates Nrf2. Blocking autophagy through chloroquine treatment results in an attenuated reduction of Keap1 by SESN2, suggesting that autophagy plays a role in decreasing Keap1expression level (40). While sestrins, AMPK, and other autophagy genes have a direct effect on Nrf2, overall metabolism has an effect on the oxidative state of the cell affecting its survival.

Cancer cells have been shown to survive far worse in nutrient poor conditions than what is needed for survival of normal cells. This relative inability of cancer cells to survive nutrient poor conditions may be due to their overreliance on metabolic pathways involving glucose. Colon cancer and breast cancer cells exhibit higher levels of glucose

consumption, PPP activity, and superoxides compared to normal counterparts, and cancer cells can compensate for increased ROS in part by increasing glucose consumption. However, when deprived of important nutrients, overwhelming levels of ROS can contribute to cell death (41).

NADPH has also been found to be important for detoxification of ROS (42, 43). Keap1-knockout MEFs have higher levels of NADPH, which is required for regeneration of reduced forms of glutathione and is a cofactor for the antioxidant protein, NQO1. Keap1-knockout MEFs also increase glucose uptake by two-fold compared to wild-type MEFs and are less able to cope with glucose withdrawal (43). It has been concluded that these cells with reduced Keap1/ activated Nrf2 are less capable of detoxifying ROS when glucose is depleted due to decreased NADPH levels, which abrogates induction of some antioxidant genes when this important co-factor is unavailable (43). Nrf2 is very important for the overall oxidative state of a cell.

2.6 Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) signaling

Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) is a transcription factor that regulates expression of cytoprotective and antioxidant genes during oxidative stress. Under normal conditions, Keap1 (kelch-like-ECH-associated protein 1) binds with Nrf2 in the cytoplasm leading to Nrf2's ubiquitination and ultimately proteasomal degradation. Oxidative stress modifies Keap1, causing the release of Nrf2 which can then translocate to the nucleus, bind to the ARE (antioxidant response element) and transcribe cyto-protective genes. Nrf2-regulated cyto-protective genes include NQO1 (NADPH quinone oxidoreductase 1) and GCLM (glutamate cysteine ligase) (44, 45). Nrf2 contributes to cell survival through several mechanisms, including upregulation of the antiapoptotic

protein Bcl2. Niture, et al. found that over-expression of Nrf2 causes an increase in Bcl2, therefore preventing death and potentially creating resistance to therapies. Knockdown of Nrf2, by contrast, was found to cause decreases in Bcl2 and NQO1, and loss of Bcl2 in turn causes cell death and DNA fragmentation, demonstrating the importance of Nrf2 in cell survival (44).

Other proteins have also been implicated in interacting with the Nrf2-Keap1 pathway. For example, p62, a protein important for bringing cargo to autophagosomes, can directly bind with Keap1 preventing it from binding to Nrf2, and thus causing its release. Especially in dysfunctional autophagy, p62 can form protein aggregates that can bind to Keap1 (46), increasing oxidative stress and leading to cytotoxicity (18). As discussed above, p62 is also involved in a paradoxical effect of autophagy where p62 aggregates may bind to and activate TRAF6, causing NFkB activation and lowering pro-apoptotic intracellular ROS, On the other hand p62 aggregates may bind caspase 8 and cause induction of apoptosis (47).

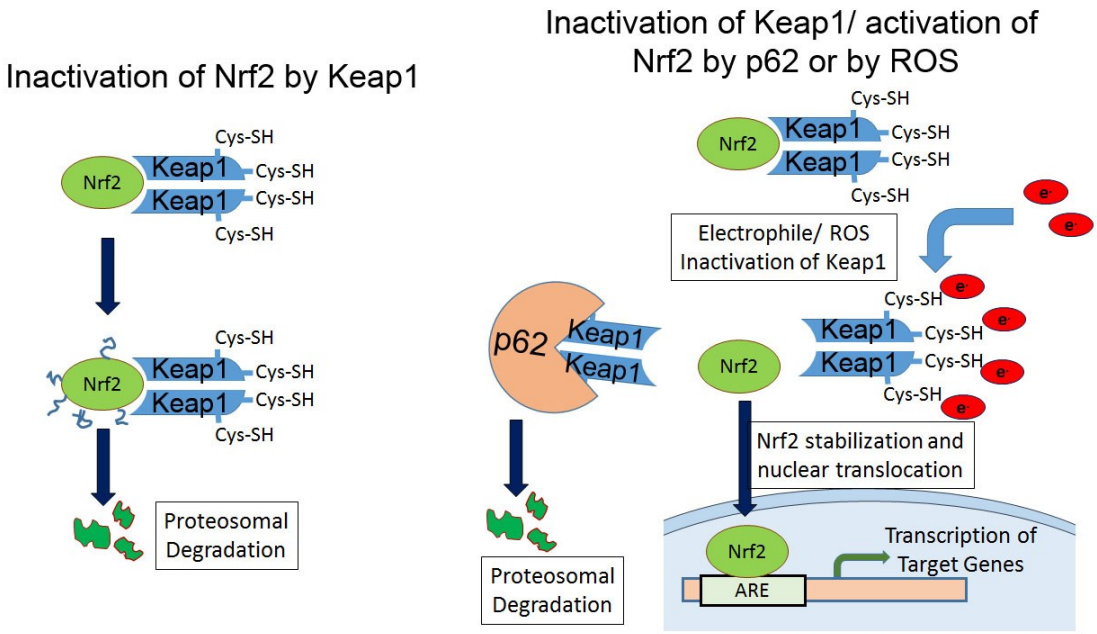

Figure 2.2 Nrf2 Pathway, adapted from "Oncogenic functions of the transcription factor Nrf2" (48)

P21 is another protein, important in cell cycle arrest, DNA repair, and apoptosis, that is upregulated in response to oxidative damage. Chen, et al. found that p21 protects cells through upregulation of the Nrf2 pathway by competing with Keap1 for binding of Nrf2. By overexpressing p21 and finding that it had no effect on survival in Nrf2-null mouse embryo fibroblasts, these investigators verified that p21 needs Nrf2 for its survival role (49) and found that p21 directly interacts with Nrf2. Nrf2 interacts with multiple other proteins and pathways in order to carry out its antioxidant function in the cell.

2.7 Nrf2 role in physiology

Nrf2 signaling is vital to managing the over-accumulation of reactive oxygen species. Type II lung cells deficient in Nrf2 have decreased proliferation and are more sensitive to oxidant induced cell death. These Nrf2-/- cells also had greater amounts of

intracellular reactive oxygen species (ROS) and low levels of the antioxidant, glutathione (GSH), and the antioxidant protein, glutamate-cysteine ligase modifier (GCLM) compared to wildtype cells. Supplementing with GSH restored proliferation to the Nrf2-null cells, however, treatment with the antioxidant NAC did not restore proliferation to the same level even though lower levels of ROS were measured with NAC treatment. The authors concluded that specifically a deficiency in Nrf2-GSH signaling impairs the type II cell growth (50). Nrf2 appears to be very important for the survival of cells by preventing ROS, and clearing the cells of ROS under oxidative stress.

2.8 Nrf2 activation in autophagy and cancer

A common denominator that involves autophagy, cancer and the Nrf2 pathway is reactive oxygen species (ROS). ROS, which are produced by mitochondria during oxidative phosphorylation, have been shown to regulate induction of autophagy; specifically, H202 oxidizes ATG4, resulting in increased formation of LC3II (36). ROS can also indirectly activate AMPK and downstream mTOR signaling (36). Cancers with activating RAS mutations have increased oxidative stress and need autophagy to tolerate this increased stress through preventing the accumulation of ROS that may damage proteins or DNA (36).

Several studies have shown that when autophagy is blocked through either chemical or genetic means, ROS increase. Wang, et.al. studied the effect of melamine, a potential carcinogen, on cell death and found it causes an increase in reactive oxygen species production as well as an increase in autophagy. By inducing autophagy using rapamycin, there was increased cell survival and decreased ROS production while conversely blocking autophagy had the opposite effect. The authors concluded that

autophagy protects PC12 cells from melamine induced death in part by clearing excess ROS (35). Excess ROS were also measured in ATG7-/- breast cancer cells, in a study by Gonzalez, et al. ATG7 knockout cells treated with MitoQ, a drug that targets the mitochondria, succumb to apoptosis whereas ATG7 wildtype cells underwent autophagy and survived, showing cells undergoing an oxidative stressor benefit from functional autophagy. Autophagy is beneficial when the cell's main mechanism of antioxidant production, the Nrf2 pathway, is not sufficient enough to combat the amount of ROS. Decreased levels of ROS and lower levels of autophagy were found in Keap1 knockout cells due to the fact that Nrf2 was activated to a high enough level to rid the cell of extra ROS without needing the additional mechanism of autophagy (37). Therefore autophagy is extremely important in a setting where the Nrf2 pathway is not functional in order to maintain a cell's survival.

2.9 Hypoxia

The tumor microenvironment not only consists of low nutrients but low levels of oxygen too as a result of poor vasculature. Cancer cells respond to the stress of hypoxia through the transcription factor HIF1 which increases glycolysis by upregulating glucose import proteins such as GLUT1 and decreases mitochondrial function by downregulating oxidative phosphorylation. Thus HIF1 helps reduce the cell's demand for oxygen while providing energy for the cell. (51)

It's interesting to note that low glucose conditions may affect ATP levels and therefore HIF1α expression. Pancreatic and prostate cancer lines exposed to low glucose suppress the increase in HIF1α levels during hypoxia. Both proteasome degradation of HIF1α and decreased transcription were ruled out as mechanisms. It was found that

glucose deprivation decreased protein synthesis which may have occurred because of decreased ATP in the cell which is needed for translation (52). Zhou also found that as HIF1α declined so did the ATP levels (53). Another study showed HIF1α levels are decreased in perinecrotic regions of tumors despite being an area that is the most hypoxic, most likely because of nutrient deprivation (54).

Presumably where hypoxia occurs, autophagy is likely to present too. Frezza et. al studied the metabolic profile of hypoxic cells and found that it had a catabolic signature. Autophagy was confirmed to be upregulated in these hypoxic cells and when autophagy was inhibited by bafilomycin A, ATP levels were depleted and significant cell death occurred. (55) Hypoxia is a stressor found in tumors that is important to study for its implication in cancer cell survival.

2.10 ER+ and HER2+ Breast Cancer

Breast cancer can be divided into 3 main categories based on receptor status: ER/PR positive, HER2 positive, and triple negative. Therapies such as Tamoxifen and Herceptin target these receptors. Approximately 50% of breast tumors rely on estrogen receptors (56). Tamoxifen prevents binding of the hormone estrogen to the estrogen receptor and prevents signaling therefore preventing growth (57, 58). HER2 is amplified in about 10% of breast cancers; this overexpression of HER2 promotes cell proliferation (56). Herceptin is a monoclonal antibody that binds to the HER2 receptor preventing HER2 signaling and therefore growth. These therapies work very well yet do not completely eradicate the cancer. Resistance can occur within a year even after an initial

response to treatment (59-61). The mechanism of resistance is important to study for future advancements in drug discovery and therapy.

Autophagy may be this mechanism, promoting cancer cell survival and preventing the therapies from effectively killing all cells. In studies performed by Qadir MA et al. cells were subjected to several conditions including tamoxifen, and siRNA for autophagy plus tamoxifen treatment. Cells treated with siRNA to the autophagy components ATG7, Beclin1, and ATG5 showed more sensitivity to tamoxifen. (59). Qadir MA also conducted studies in which cells were incubated with tamoxifen for 72 hours and visualized for LC3 by fluorescent microscopy. During Tamoxifen treatment, the GFP-LC3 became punctate indicating autophagosomes were forming in response to tamoxifen (59). As a result, the efficacy of targeted drug therapies may be increased by targeting autophagy.

While Qadir MA showed that autophagy may be a factor in drug efficacy, Nahta R et al. found that when SKBR3 parental cells are compared to trastuzumab resistant pools of cells, they had equal amounts of HER2 protein. However when the pools were maintained in trastuzumab there was a downregulation of HER2 in these resistant cells compared to the parental cells where HER2 did not change (62). Vazquez-Martin et al also studied the trastuzumab refractory cells and found that autophagy was upregulated in these resistant pool compared to naïve cells (63). With this information, I came to the hypothesis that metabolic stress-induced autophagy downregulates ER and HER2 in breast cancer cells, resulting in an attenuated response to targeted drug therapies. This hypothesis was explored and while seemingly not correct, produced some interesting data that will be discussed in chapter 6.

Chapter 3 Induction of Autophagy by Glucose Deprivation

3.1 INTRODUCTION

In human tumors, cancer cells often encounter ischemia and require activation of various cell survival mechanisms to withstand this metabolic stress. Autophagy is one important potential mechanism for providing energy and essential nutrients during metabolic stress, but the role of autophagy during nutrient deprivation – and specifically glucose deprivation - has been an area of dispute (64). In multiple experiments, knockdown of genes essential for autophagy in cells deprived of serum or amino acids was found to lead to cell death by apoptosis (65, 66), and autophagy has also been shown to protect cancer cells from the glycolytic inhibitor 2-deoxyglucose (2-DG) (67-69). While incubating cells with 2-deoxyglucose might be expected to mimic the situation of glucose deprivation, experiments testing glucose free medium in a variety of cultured cell lines, did not find a protective role for autophagy in the setting of glucose deprivation (70).

Cellular responses to glucose deprivation are probably variable among cell types with different molecular backgrounds, and in addition to the process of autophagy, other cellular adaptations likely have complementary or parallel role for cell survival during metabolic stress. Other sections of this thesis will describe my work on how induction of Nrf2 activity is also critical for survival of cells during glucose deprivation, which was an unexpected finding because previous studies have found that autophagy leads to reduced levels of p62 (33, 46, 71), which in turn would be expected to allow Keap1 to bind the Nrf2 transcription factor and lead to increased degradation of Nrf2 protein.

3.2 MATERIALS AND METHODS

3.2.1 Cell Culture

Human cancer cell line MCF7 was cultured in DMEM media (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). T47D was cultured in RPMI (Gibco) supplemented with 10% FBS. Experiments requiring glucose deprivation used RPMI glucose free media (Gibco) 0.1%FBS. DMEM glucose free media was made from DME base (Sigma Aldrich) dissolved in ultrapure water and supplemented with sodium bicarbonate, sodium pyruvate, L-glutamine and 0.1%FBS.

3.2.2 Reagents

Primary antibodies for ATG7, Beclin1, cleaved PARP (Asp214), and PARP were purchased from Cell Signaling Technology. LC3B was purchased from Novus Biologicals. p62 antibody was purchased from Santa Cruz. Anti-Actin antibody was purchased from Sigma-Aldrich. Anti-rabbit and anti-mouse secondary antibodies were purchased from Cell Signaling Technology. Chloroquine, a pharmacological inhibitor of autophagy, was dissolved in water to make a 25mM stock concentration. Puromycin was purchased from Sigma and dissolved in ultrapure water to make a 10mg/ml stock solution. Alexa Fluor 488 Donkey anti-rabbit IGg and Alexa Fluor 594 Goat anti-mouse IGg were purchased from Invitrogen. Prolong Gold antifade reageant with DAPI by Molecular Probes Life Technologies was used to stain the nucleus in immunofluorescence experiments.

3.2.3 Clonogenic Assays

MCF7 and T47D were plated at appropriate density ranging from 500 to 3000 cells per well. After 24 hours incubation, cells were treated with complete or glucose free medium for variable amounts of time with the most common being 72 hours. In other experiments, cells were treated with complete or glucose free media long with variable concentrations of chloroquine according to the experiment. After treatment with the different mediums and drugs, cells were replenished with complete media without drug and allowed to grow for an average time of 10 days before being fixed with 100% methanol and stained with 0.5% crystal violet (Sigma).

3.2.4 Immunofluorescence

MCF7 cells were plated at a density of 1500 per chamber slide well. After incubation with complete or glucose free media and for 48 hours, slides were washed with PBS and fixed for 10 minutes in Zn Formalin. Cells were permeablized with .1% Triton-PBS for 10 minutes and then blocked with 5%BSA in PBS for 1 hour before adding primary antibody (1:200 dilution) and incubating overnight at 4ºC. The slides were then washed 3 times for 10 minutes each and then incubated with secondary antibody (1:200 dilution) (Alexa Fluor 488 Donkey anti-rabbit IGg and Alexa Fluor 594 Goat anti-mouse IGg) for 1 hour in the dark. Slides were washed again and DAPI was added to the slide and covered with a coverslip. Images were taken using Nikon eclipse 50i and the program NIS-Elements BR 3.2.

3.2.5 Immunoblot

After experimental treatment, cells were harvested in TNE lysis buffer plus protease inhibitor and protein concentration was determined using Pierce BCA assay (Thermo Fisher Scientific). Equal amounts of protein were loaded for each sample, separated by SDS-PAGE on a 10% or 10-20% gel Tris-HCl gel (Bio-Rad) and incubated overnight with primary antibody in 4ºC. Most antibodies were incubated at a 1:1000 concentration. Exceptions include actin (1:20,000), and sigma antibodies (1:500). After washing and a 1.5 hour incubation with anti-rabbit or anti-mouse secondary antibody at room temperature, membranes were developed with SuperSignal West Femto Max Sensitivity Substrate (Thermo Fisher Scientific). Some membranes were stripped with Stripping Buffer (Thermo Fisher Scientific) and re-probed with other primary antibodies.

3.2.6 Flow Cytometry

T47D and MCF7 cells were grown to sub-confluency in 100mm cell culture plates and grown in complete or glucose free media for 72 hours. At the designated time, cells were trypsinized with 0.25% Trypsin-EDTA, collected and then washed with PBS. Cells were resuspended in 0.3ml PBS and fixed with 5ml of a 1:1 methanol:acetone mixture. Fixed cells were centrifuged for 5 minutes at 2,000 RPM and buffer decanted. The cell pellet was suspended in 5ml of PBS, and then centrifuged for 5 minutes at 2,000 RPM. After aspirating PBS, 0.25ml of 5ug/ml RNase was added with a wait time of 15 minutes at 37C. Next 0.25ml of 100ug/ml PI solution was added for an incubation time of overnight. Fixed cells were stained with Propidium Iodide (Sigma) for 1 hour. Propidium Iodide fluorescence of the samples was determined by FACS.

3.2.7 CRISPR/Cas-9 Transfection

5ug of lentiCRISPRv2 plasmid (Plasmid #52961) was digested and dephosphorylated with BsmB1 for 30 minutes at 37ºC. The digested plasmid was purified using QIAquick gel extraction kit and eluted in distilled water. Next, oligos for ATG7 and Beclin 1 were phosphorylated by T4 poly nucleotide kinase and annealed at 37ºC for 30 minutes, followed by 95ºC for 5 minutes and ramping down to 25ºC at 5C/minute. Digested lentivirus and annealed oligos were followed by a ligation reaction and incubated at room temperature for 10 minutes. The ligated DNA were transformed into Stbl3 bacteria and positive colonies were selected on ampicillin contained LB agar media and cultured in ampicillin contained LB media. Supernatant was harvested and DNA was then purified using Invitrogen PureLink HiPure Plasmid Filter Midiprep Kit. Correct insertion was verified using sequencing. lentiCRISPR with inserted sequences were co-transfected into HEK293T cells with packinging plasmids pVSVg and psPAX2. MCF7 cells were then transfected twice, allowed to grow for 3-4 days cells and selected for a week. Cells were diluted into a 96 well plate and single colonies were picked. Knockdown of ATG7 and Beclin1 were verified by immunoblot and Cas9 antibody. Knockdown cells were maintained in DMEM with 1ug/ml of puromycin. lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52961) (72)

GENE	REFERENCE SEQUENCE	OLIGONUCLEOTIDE SEQUENCE
ATG7	NM_006395.2	Forward 1: 5'CACCGAATCAAGTATGATGAGAACA3'
		Reverse 1: 5'AAACTGTTCTCATCATACTTGATTC3'
		Forward 2: 5'AAACTGTTCTCATCATACTTGATTC3'
		Reverse 2: 5'AAACGGACGACTCACAGTGCACTGC3'
Beclin1	NM_003766.3	Forward 1: 5'AAACGGACGACTCACAGTGCACTGC3'
		Reverse 1: 5'AAACGCATGGTGCTGTTGTTGGACC3'
		Forward 2: 5'CACCGGCCAACAGCTTCACTCTGAT3'
		Reverse 2: 5'AAACATCAGAGTGAAGCTGTTGGCC3'

Table 3.2.1 Oligonucleotide sequences for CRISPR

3.3 RESULTS

3.3.1 Glucose deprivation results in the induction of autophagy in cultured breast cancer cells

Although autophagy is generally believed to have an essential role in supporting cell survival during nutrient deprivation, conflicting data has been presented regarding the role of autophagy in the setting of glucose deprivation. Accordingly, we cultured MCF7 and T47D breast cancer cells in glucose-free medium for variable periods, and to measure effects of the nutrient deprivation on cell survival, we then replenished nutrients with full media for seven days and measured clonal growth of surviving cells. Accordingly, we cultured MCF7 and T47D breast cancer cells in glucose-free medium to test for cell viability over several days. As seen in Figure 3.1A cell death occurs noticeably at 72 hours. To further assess cell viability at 3 days of glucose deprivation, we cultured MCF7 and T47D, in glucose-free media for 3 days and replenished nutrients with complete media for 10 days to measure clonal growth of surviving cells. Both cell lines generally tolerate up to approximately three days of glucose-free media (Fig 3.1B, and Fig 3.1C).

To determine whether autophagy is induced by this glucose deprivation, we measured conversion of the microtubule-associated protein light chain 3 protein (LC3) from the LC3I form to the LC3II form, which is considered to be characteristic of autophagy. As seen in Figure 3.2, the ratio of LC3II:LC3I increases with glucose deprivation in both T47D and MCF7, particularly after the addition of chloroquine, which raises lysosomal pH and prevents fusion and degradation of lysosomal proteins, thus enhancing detection of increases in LC3II. Although actin loading for 72 hours of glucose deprivation is unequal, presumably the LC3II levels in the chloroquine treated

lane would be further increased, still supporting an increase in autophagy. Glucose deprivation for two days also results in the appearance of a punctate staining pattern of LC3II, which indicates the presence of autophagosomes typical for autophagy (Fig 3.3).

3.3.2Pharmacologically blocking autophagy decreases survival of breast cancer cells during glucose deprivation

We then tested the significance of autophagy in survival of breast cancer cells during glucose deprivation. First, we tested how chloroquine treatment, which blocks completion of autophagy, affects survival. As seen in Figure 3.4A , addition of chloroquine at levels that alone do not affect cell growth do cause loss of cell viability, when T47D cells are grown in glucose-free conditions for three days. Inhibition of autophagy during glucose deprivation using chloroquine similarly results in loss of cell viability in MCF7 cells (Fig 3.4B). As reported in other experimental systems, cell death in this setting is associated with changes typical for apoptosis, including cell fragmentation (Fig 3.4A) and increases in cleaved poly-ADP-ribose polymerase (Fig 3.5).

3.3.3Knockout of genes required for autophagy also decreases survival of breast cancer cells during glucose deprivation

To more specifically measure the role of autophagy in cell survival, we then used a CRISPR-Cas9 approach to knock out two genes that are critical for autophagy: ATG7 and beclin-1. Data shown in Figure 3.6A demonstrates that this strategy results in the complete knockout of expression of these targeted genes in MCF7 cultures, and Figure

3.6B shows that knockout of expression of these genes results in greatly reduced survival after glucose deprivation. Survival of cells transfected with a control vector are unaffected and retain the ability to survive three days of glucose deprivation, and knockout of either ATG7 or beclin-1 does not affect survival of cells cultured with full media. Thus, glucose deprivation leads to induction of autophagy in breast cancer cells, and this autophagy contributes to survival of cells for several days of glucose deprivation.

3.3.4Autophagy is responsible for reduced levels of p62 during glucose deprivation

Previous studies demonstrated that autophagy can lead to degradation of endogenous p62 or ectopic expression of p62, and in accordance with these previously reported findings, we also observed decreased levels of p62 in cultures of MCF7 cells at 48 hours or 72 hours of glucose deprivation, when compared to control cultures with full glucose media (Fig 3.7A and Fig 3.7B). Decreases in levels of p62 were evidently dependent on autophagy, since p62 levels did not decrease in cells that had ATG7 or beclin 7 knock-out (Fig 3.7C).

3.4 DISCUSSION

In our studies using breast cancer cells, glucose deprivation resulted in autophagy, which in turn allowed survival of the cells for approximately three days in these starvation conditions. Autophagy was demonstrated in our experiment by measuring the LC3II : LC3I ratio and visualizing the induction of punctate LC3 foci in cells during glucose deprivation. The importance of autophagy in cell survival was demonstrated by reduced clonogenicity when autophagy was obstructed by pharmacological agents such as chloroquine or by knockout of proteins that are key in the autophagic process.

Autophagy has been shown to be critical for cell survival in many settings of cellular stress (73) including ER stress, hypoxia, redox stress, mitochondrial damage and metabolic stress. However, the role of autophagy in cell survival during nutrient deprivation- and specifically glucose deprivation- has been an area of dispute (64). Knockdown of essential autophagic genes in cells deprived of serum or amino acids lead to cell death by apoptosis and autophagy has been shown to protect cancer cells from 2-deoxyglucose, a glycolytic inhibitor, which could represent a metabolic setting similar to glucose deprivation. In experiments that directly tested glucose deprivation, however, autophagy reportedly did not protect a variety of cell lines culture in this setting.

Our experiments provide a direct demonstration that in MCF7 and T47D breast cancer cells, autophagy is induced by glucose deprivation and is critical for survival of these cells in this setting for up to three days. While differences between our results and those previously reported for glucose deprivation cannot be easily explained, we note that almost certainly, cellular responses to glucose deprivation will vary among different cell types with different molecular backgrounds. This may be especially true in cancer cells.

Indeed, some cancer cells are highly dependent on glutamine as their energy source and for these cells, glucose deprivation might be of little consequence. Moreover, as I will show later in this thesis, other cellular protective mechanisms, including Nrf2, might be critical for survival in the setting of glucose deprivation.

While autophagy is important for providing critical nutrients and energy during metabolic stress, one of its key players, p62, an LC3-interacting, ubiquitin associated protein, is involved with Nrf2 signaling. When autophagy is dysfunctional, p62 has been shown to interact with Keap1, sequestering it, which in turn leads to induction of antioxidant Nrf2 signaling. Our studies have shown that, as expected, autophagy induced by glucose deprivation reduces levels of p62. Presumably in this setting, levels of Keap1 would increase, thus enabling the ubiquitination and degradation of Nrf2. In the next chapter we'll explore whether this interaction between Keap1 and p62 affects Nrf2 signaling in glucose deprivation.

The induction of autophagy and the protective effects of this process during glucose deprivation could be important for survival of cancer cells in situation of metabolic stress, such as ischemia. In fact, substantial date points to autophagy contributing to cancer development in a variety of experimental settings. Particularly in cancers with RAS mutations, experimental data indicates that autophagy is critical for growth and survival of cancer cells. In most cancerous tumors, the disordered microenvironment is associated with metabolic stress due to variable hypoxia and nutrient insufficiency, and in this setting autophagy appears to have an essential role in recycling intracellular macromolecules and organelles to transiently provide essential metabolic substrates in such settings.

3.5 FIGURES

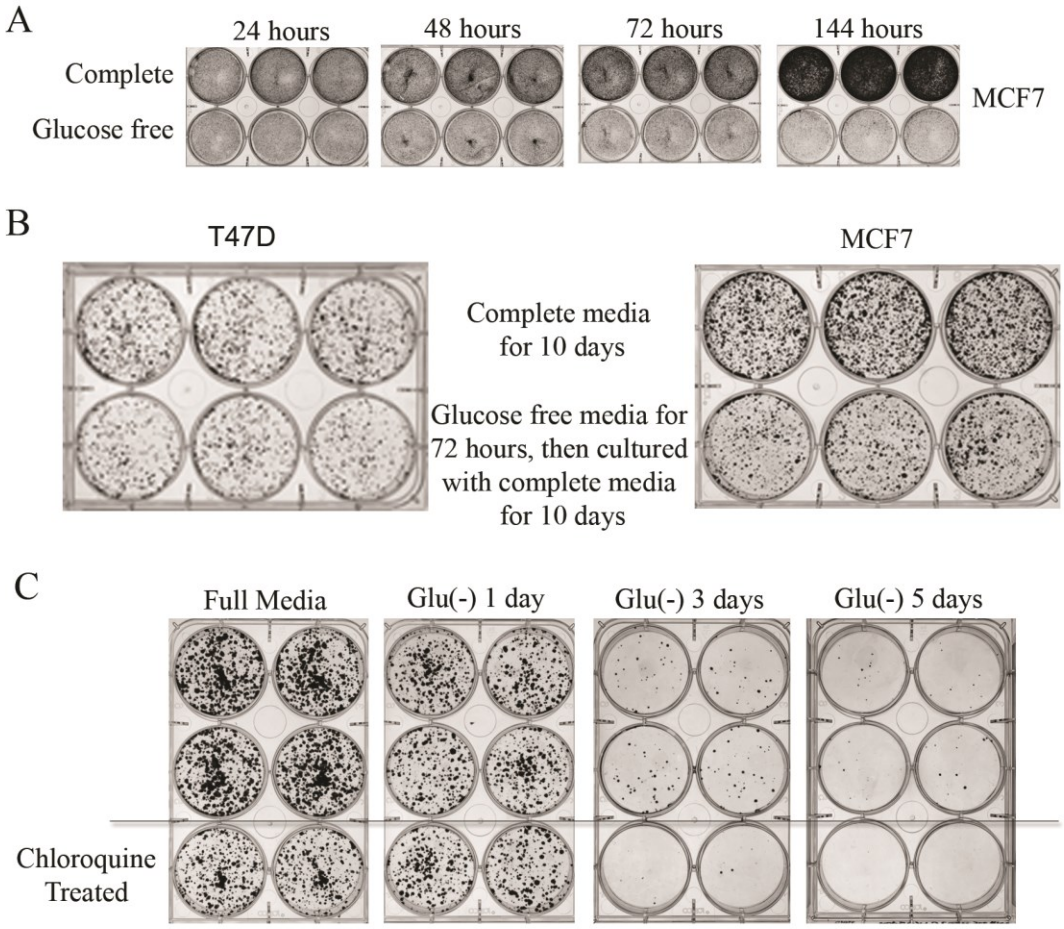

Figure 3.1 Breast cancer cells survive nutrient deprivation. (A) MCF7 cells were cultured in complete or glucose free media for 24, 48, 72, or 144 hours and then fixed with MeOH and stained with crystal violet. Cells are tolerant of glucose deprivation for several days. (B) After attachment, T47D and MCF7 cells were cultured in either complete media (top row) for a total of 10 days, or glucose free media (bottom row) for 72 hours followed by complete media for an additional 10 days. Cells were fixed and stained with crystal violet. (C) MCF7 cells were cultured in complete or glucose free media for 1, 3 or 5 days after which they were replenished with complete media for 10 days. Bottom two wells were treated with 7uM chloroquine. When colonies were visible plates were stained with crystal violet.

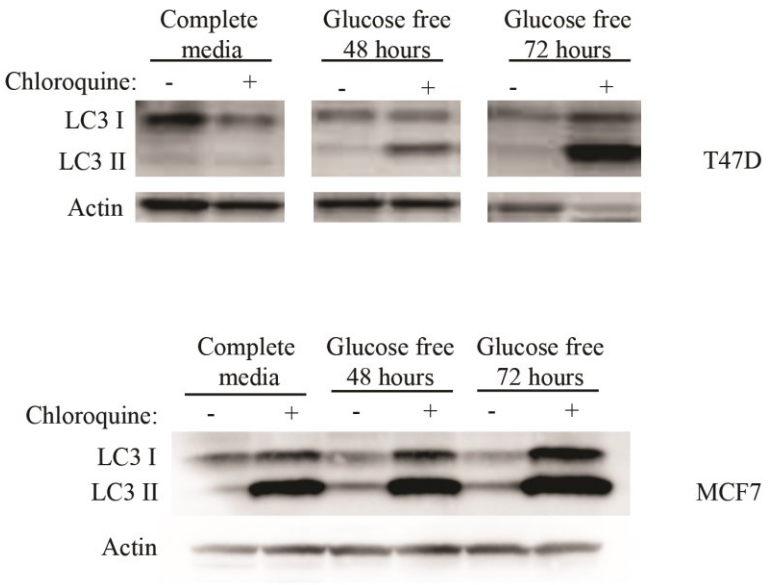

Figure 3.2 Breast cancer cells induce autophagy during glucose deprivation. T47D and MCF7 cells were grown in complete media or glucose free media for 48 or 72 hours and assayed for LC3 by immunoblot. Cells were treated with 25uM chloroquine to assess autophagic flux. Increased levels of the LC3II form of the protein, characteristic of autophagy, is seen in glucose deprivation conditions, and the addition of chloroquine, which blocks completion of autophagy, enhances the detection of increased LC3II.

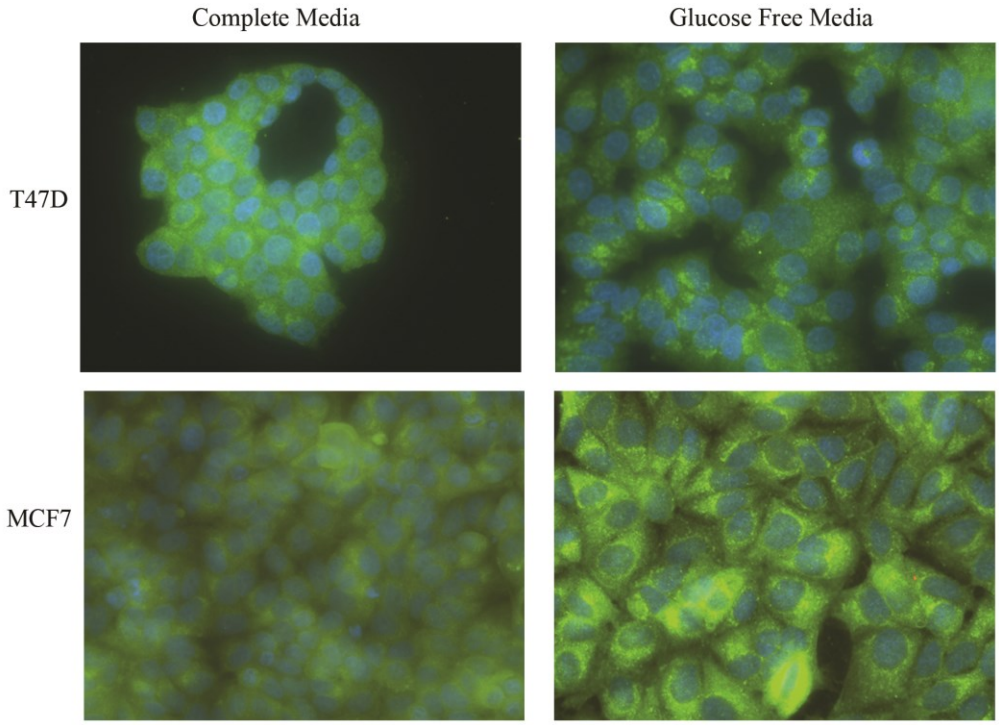

Figure 3.3 Punctate autophagosomes are increased during glucose deprivation. Breast cancer cell lines, T47D and MCF7, were grown in either complete media or glucose free media for one day, and LC3II was visualized using immunofluorescence. In contrast to the diffuse, green LC3II staining of breast cancer cells grown in complete media, punctate staining is seen when cells are cultured in glucose deprivation, typical of autophagosomes. (green=LC3, blue=DAPI)

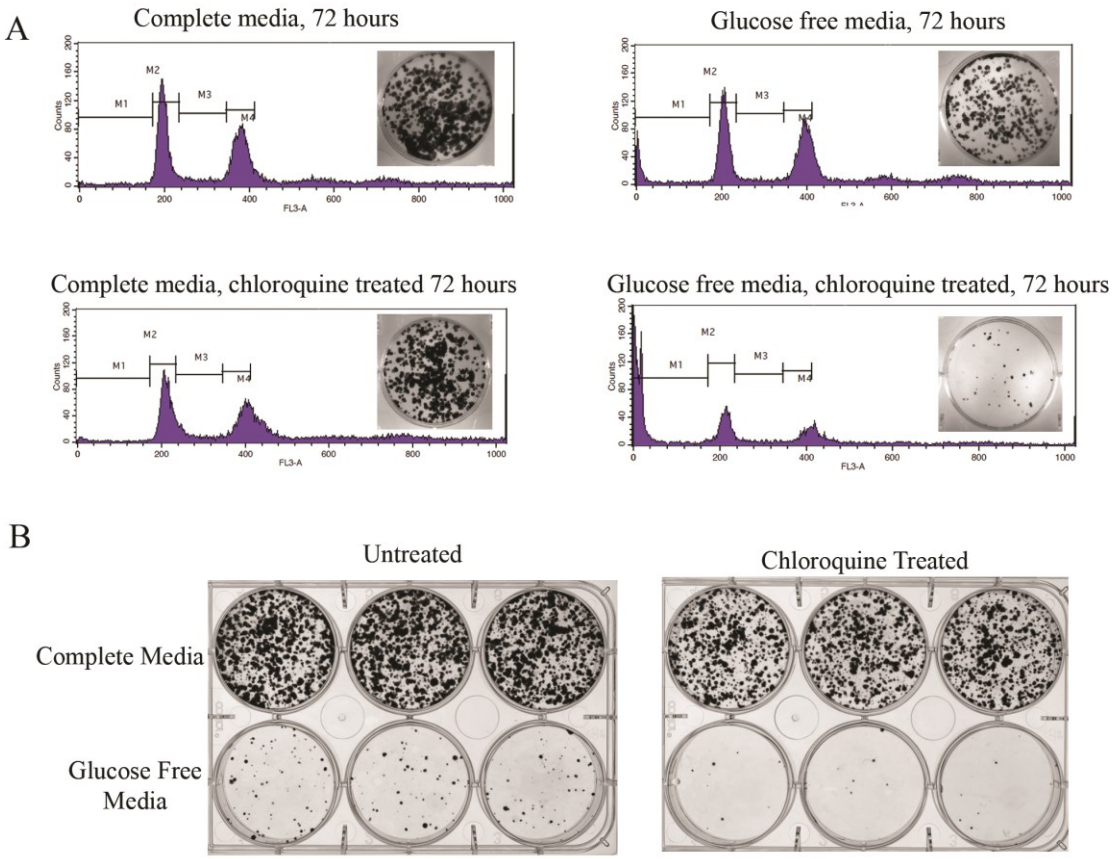

Figure 3.4 Autophagy is essential for survival during nutrient deprivation. (A) T47D cells were analyzed for cell cycle by flow cytometry. All treatments were carried out for 72 hours, then cells were fixed. Chloroquine (25uM), an autophagic inhibitor, had little effect on cells grown in complete media. Cells grown in glucose free media have an increased subG1 population. Cells grown in glucose free media and treated with chloroquine for 72 hours have an even larger increase in subG1 population. The inset on each flow diagram consists of T47D cells plated in 6 well plates and subjected to the same conditions shown by FACs. After 72 hours, all cells were grown in complete media to allow formation of colonies to be stained by crystal violet. (B) MCF7 cells were treated with complete or glucose free media with or without chloroquine (7uM) for 72 hours, replenished and stained with crystal violet.

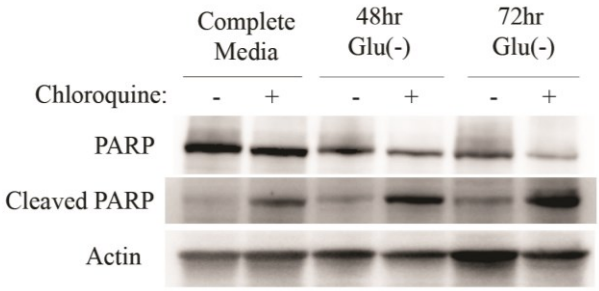

Figure 3.5 MCF7 cells undergo apoptosis when autophagy is blocked during glucose deprivation. MCF7 cells were grown in complete media or glucose free media for 48 or 72 hours with or without 25uM chloroquine and assayed for cleaved PARP by immunoblot.

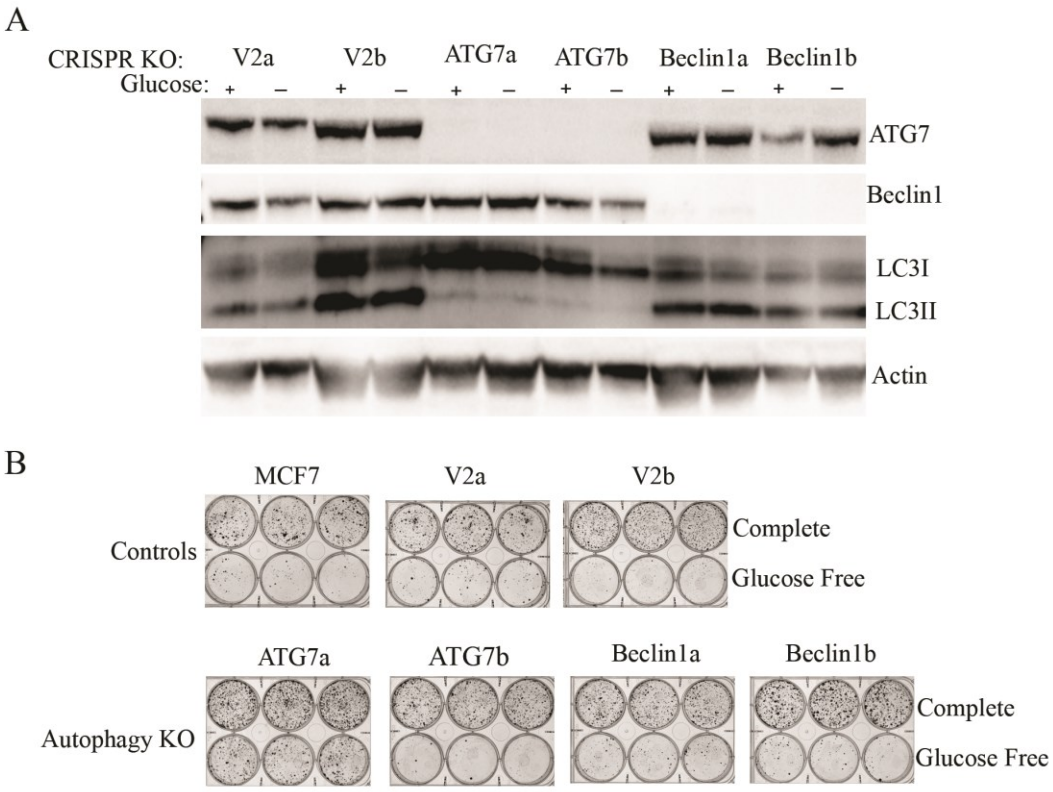

Figure 3.6 Genetic knockout of autophagic proteins decreases survival (A) MCF7 cells were transfected with CRISPR constructs ATG7, Beclin1, and a control vector V2. Knockout of ATG7 and Beclin1 were achieved. (B) MCF7 CRISPR cell lines were grown in complete or glucose free media for 72 hours followed by complete media for 7 days. Cells were stained with crystal violet.

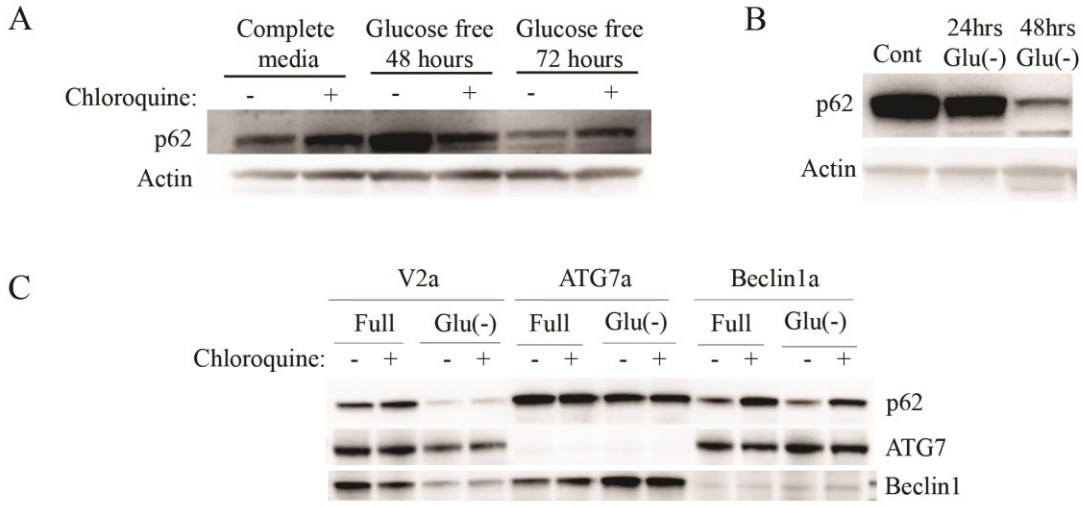

Figure 3.7 p62 is decreased during glucose deprivation. (A) MCF7 cells were grown in complete media or glucose free media for 48 or 72 hours with or without 25uM chloroquine and assayed for p62 by immunoblot. (B) MCF7 cells were grown in complete media or glucose free media for 48 or 72 hours and assayed for p62 by immunoblot. (C) Cells were grown in complete or glucose free media with or without 7uM chloroquine and harvested 24 hours later. P62, ATG7 and Beclin1 were assayed by immunoblot.

Chapter 4 Induction of Nrf2 by Glucose Deprivation

4.1 INTRODUCTION

The previous section of this thesis describes our findings that, a) cultured breast cancer cells are capable of surviving glucose deprivation for several days, b) autophagy is induced by glucose deprivation results in these breast cancer cells, and c) autophagy is critical for cell survival in this setting. Since other cellular adaptations likely have complementary or parallel role for cell survival during metabolic stress, we investigated what protective molecular pathways might be triggered by autophagy in the setting of glucose deprivation. In particular, my studies focused on Nrf2 signaling and its tie to autophagy through p62, an LC3-interacting, ubiquitin-associated protein. In various disease settings, p62 accumulates in cytosolic protein aggregates and in cellular inclusion bodies together with polyubiquitinated proteins. Autolysosomes containing p62- and LC3-positive bodies are degraded by autophagy (74), resulting in decreased levels of p62.

Consequently, in previous experiments and as presented in Chapter 3, autophagy was found to result in reduced levels of p62, (33, 46, 71). Interestingly, p62 has been shown to sequester Keap-1 protein (74, 75), however if p62 levels are down, it would be expected to allow Keap1 to bind the Nrf2 transcription factor and lead to increased degradation of Nrf2 protein.

This chapter will describe my unexpected finding that Nrf2 protein levels and Nrf2 activity are actually *increased* in the setting of glucose deprivation, which is an unforeseen finding in light of previously reported findings that decreases in p62 lead to decreases in Nrf2. I will describe how this increase in Nrf2 is independent of autophagy, and also independent of reactive oxygen species, which do increase in glucose-deprived

cells. In addition, I'll present my findings from testing the role of Nrf2 in cell survival during glucose deprivation.

4.2 MATERIALS AND METHODS

4.2.1 Cell Culture

Human cancer cell line MCF7 was cultured in DMEM media (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). T47D was cultured in RPMI (Gibco) supplemented with 10% FBS. Experiments requiring glucose deprivation used RPMI glucose free media (Gibco) 0.1%FBS. DMEM glucose free media was made from DME base (Sigma Aldrich) dissolved in ultrapure water and supplemented with sodium bicarbonate, sodium pyruvate, L-glutamine and 0.1%FBS. MCF7shRNA cells were maintained in DMEM with 750ng/ml of puromycin.

4.2.2 Reagents

Primary antibodies for NQO1, cleaved PARP (Asp214), and PARP were purchased from Cell Signaling Technology. GCLM was purchased from Novus Biologicals. p62, and Nrf2 antibodies were purchased from Santa Cruz. Anti-Actin antibody was purchased from Sigma-Aldrich. Anti-rabbit and anti-mouse secondary antibodies were purchased from Cell Signaling Technology. Chloroquine, a pharmacological inhibitor of autophagy, was dissolved in water to make a 25mM stock concentration. Glutathione (GSH) (Sigma) was dissolved in water to make a 50mM stock concentration. N-acteyl-cysteine (NAC) (Sigma) was prepared in water to make a 500mM stock concentration. Puromycin was purchased from Sigma and dissolved in ultrapure water to make a 1mg/ml stock solution. ML-385, Nrf2 inhibitor, was provided by Anju Singh in the Biswal Lab and dissolved in DMSO for a stock concentration of 10mM. Alexa Fluor 488 Donkey anti-rabbit IGg and

Alexa Fluor 594 Goat anti-mouse IGg were purchased from Invitrogen. Prolong Gold antifade reageant with DAPI by Molecular Probes Life Technologies was used to stain the nucleus in immunofluorescence experiments. CM-H2DCFDA was purchased from Molecular Probes Life Technologies.

4.2.3 Immunoblot

After experimental treatment, cells were harvested in TNE lysis buffer plus protease inhibitor and protein concentration was determined using Pierce BCA assay (Thermo Fisher Scientific). Equal amounts of protein were loaded for each sample, separated by SDS-PAGE on a 10% or 10-20% gel Tris-HCl gel (Bio-Rad) and incubated overnight with primary antibody at 4ºC. Most antibodies were incubated at a 1:1000 concentration. Exceptions include actin (1:20,000), and sigma antibodies (1:500). After washing and a 1.5 hour incubation with anti-rabbit or anti-mouse secondary antibody at room temperature, membranes were developed with SuperSignal West Femto Max Sensitivity Substrate (Thermo Fisher Scientific). Some membranes were stripped with Stripping Buffer (Thermo Fisher Scientific) and re-probed with other primary antibodies.

4.2.4 Immunofluorescence

MCF7 shRNA cell lines were plated at a density of 1500 per chamber slide well. After incubation with complete or glucose free media for 48 hours, slides were washed with PBS and fixed for 10 minutes in Zn Formalin. Cells were permeablized with .1% Triton-PBS for 10 minutes and then blocked with 5%BSA in PBS for 1 hour before adding primary antibody and incubating overnight at 4ºC. The slides were then washed 3 times

for 10 minutes each and then incubated with secondary antibody (Alexa Fluor 488 Donkey anti-rabbit IGg and Alexa Fluor 594 Goat anti-mouse IGg) for 1 hour in the dark. Slides were washed again and DAPI was added to the slide and covered with a coverslip. Images were taken using Nikon eclipse 50i and the program NIS-Elements BR 3.2.

4.2.5 shRNA Transfection

MCF7 cells were transfected with Keap1 shRNA, Nrf2 shRNA and control luciferase shRNA. Lentivirus was supplied by Anju Singh. Cells were plated at 30-40% confluence. 8ug/ml of polybrene was added to viral supernatant and incubated for 10 minutes at room temperature. This mixture was mixed with fresh media and added to the cells and incubated overnight. The next day the culture medium with polybrene and lentivirus was replaced with fresh media. Cells were selected with 750ng/ml of puromycin.

4.2.6 q-PCR/quantitative PCR

After transfection, MCF7 Nrf2shRNA, MCF7 Keap1shRNA, and MCF7 LucshRNA cells were analyzed for mRNA levels of the transcripts for Nrf2, Keap1, NQO1, and Gclm to ensure transfection was efficient. 1ug of RNA was used for cDNA synthesis using ABI high capacity cDNA reverse transcription kit. cDNA synthesis was carried out on regular thermocycler.

1ug RNA in10ul (volume adjusted with water)	10ul
Buffer	2ul
Primers	2ul
dNTP	0.8ul
Multiscribe reverse transcriptase	1ul
Water	4.2ul
	Total Volume= 20ul

Table 4.2.1 RT-PCR/cDNA synthesis mixture

25ºC	10 minutes
37ºC	2 hours
85ºC	10 minutes

Table 4.2.2 RT-PCR cDNA synthesis cycling conditions

The cDNA was then diluted 5 times in water, and used for real time PCR using Taqman primer/probe mix and ABI gene expression mastermix.

2X mastermix	10ul
20X Taqman primer/probe mix	1ul
cDNA	5ul
Water	4ul

Table 4.2.3 qPCR reaction mixture

1 cycle	95ºC for 20 seconds
40 cycles	95ºC for 1 second, 60ºC for 20 seconds

Table 4.2.4 qPCR cycling conditions

GENE	TAQMAN PRIMER PROBE ID
NQO1	Hs00168547_m1
GCLM	Hs00157694_m1
Nrf2	Hs00975961_g1
Keap1	Hs00202227_m1

Table 4.2.5 Taqman primer probe sequences for qPCR

Statistical significance was determined by upaired t-test in GraphPad Prism4 from GraphPad Software, Inc., La Jolla, CA.

4.2.6 Clonogenic Assays

MCF7 and T47D were plated at appropriate density ranging from 500 to 3000 cells per well. After 24 hours incubation, cells were treated with complete or glucose free medium for variable amounts of time with the most common being 72 hours. In other experiments, cells were treated with complete or glucose free media with 2uM ML-385, 2mM NAC, or 2.5mM GSH according to the experiment. After treatment with the different mediums and drugs, cells were replenished with complete media without drug and allowed to grow for an average time of 10 days before being fixed with 100% methanol and stained with 0.5% crystal violet (Sigma).

4.2.7 ROS Measurements

MCF7 were plated at a density of 7000 -10,000 cells per well in a 96 well plate. After 24 hours incubation, media was changed to complete DMEM or DMEM glucose free media. At 12 and 24 hour time points, cells were aspirated and washed with PBS. CM-H2DCFDA, dissolved in ethanol to a final concentration of 20uM in PBS, was added to the wells and incubated for 30 min. Cells were then aspirated, and 10%FBS in PBS was

added and incubated for 30 min. Wells were aspirated and fluorescence was measured using Filter Max F5 (Multi-mode Microplate Reader, Molecular Devices) and Soft Max Pro 6.3 software at an excitation of 495 and emission of 520. Statistical significance was determined by upaired t-test in GraphPad Prism4 from GraphPad Software, Inc., La Jolla, CA.

4.3 RESULTS

4.3.1The Nrf2 transcription factor is unexpectedly induced in breast cancer cells during nutrient deprivation

As noted above, in the presence of p62, Nrf2 levels increase, but in the absence of p62, Nrf2 levels would be expected to decrease. Interestingly, Nrf2 activity is often activated in human cancers, leading us to question how autophagy might affect this pathway in cancer. We first tested whether Nrf2 is decreased by nutrient deprivation in breast cancer cells by measuring levels of Nrf2. As shown in Figure 4.1A, levels of Nrf2 surprisingly increase in MCF7 cells when cultured in the absence of glucose. Induction of Nrf2 was also confirmed using immunofluorescence to measure the levels of the Nrf2-regulated transcript, NADPH dehydrogenase quinone1 (NQO1), a major antioxidant protein. As seen in Figure 4.1B, cells cultured with glucose deprivation conditions have increased NQO1 expression compared to control cultures.

4.3.2Induction of the Nrf2 transcription factor is critical for survival of breast cancer cells during nutrient deprivation-induced autophagy

Although activation of Nrf2 is glucose deprivation was unexpected, based on previously published experiments, we noted that Nrf2 activity is often activated in human cancers and is an important protective of cells in response to a variety of stresses. The role of Nrf2 thus led me to question how increased Nrf2 might affect survival in the setting of glucose deprivation.

To test whether this unexpected increase in Nrf2 protein and activity has a role in survival of glucose-deprived breast cancer cells, we transfected shRNA designed to knock-down levels of Keap1 or Nrf2 transcripts. qPCR was performed to determine that

the transfection was successful. As shown in Figure 4.2A both Nrf2 and Keap1 were knocked down and increases of Nrf2-regulated mRNA transcripts was increased in the Keap1 knockdown cells as expected. Furthermore, the shRNA targeting Keap1 increases levels of Nrf2-regulated antioxidant proteins NQO1 and Gclm , whereas levels of these proteins show minimal to no decrease by shRNA targeting Nrf2 when compared to luciferase control (Fig 4.2B) .

Examining survival of cells using clonal growth assays either in full media or glucose deprivation, I found that cells depleted of Keap1 show only a modestly improved survival compared to control cultures (Fig 4.3A and Fig 4.3B). This result likely reflects our previous observation that Nrf2 levels are increased by glucose deprivation alone, even without knockdown of Keap1, and thus knockdown of Keap1 by shRNA likely has minimal additive effect. More remarkably, however, cells depleted of Nrf2 have significantly impaired survival after glucose deprivation compared to cells that were transfected with a control luciferase shRNA, demonstrating that Nrf2 has a critical role in survival of these cells during glucose deprivation (Fig 4.3A and Fig 4.3B). The importance of Nrf2 was also investigated using the Nrf2 inhibitor ML-385. Treatment of both autophagy-competent and autophagy-deficient cells with 2uM ML-385 resulted in increased cell death in glucose free media but not complete media when compared to the untreated control plate (Fig 4.4). Thus, our experiments find that the increased Nrf2 expression and activity in the setting of glucose deprivation have a critical role in survival of breast cancer cells in this setting.

4.3.3Glucose deprivation results in increased levels of reactive oxygen species, and protective effects of Nrf2 are mediated by antioxidant activity

As demonstrated above as well as in previously reported studies, Nrf2 activity regulates levels of the antioxidant protein, NADPH dehydrogenase quinone 1 (NQO1), resulting in protection from reactive oxygen species (ROS). To explore how glucose deprivation and Nrf2 activity affect cellular levels of ROS, we used 2',7'-dichlorodihydrofluorescein diacetate (DCFDA), a cell-permeable, chemically reduced form of fluorescein, as an indicator for ROS in cells. As shown in Figure 4.5A, cultures of cells with knockdown of Nrf2 showed time-dependent increases in ROS when cultured in glucose-free medium. By contrast, cells with knockdown of Keap1 showed significantly less ROS when cultured in glucose-free medium. These results show that cellular levels of ROS are increased during glucose deprivation, and that Nrf2 (especially when induced by knockdown of Keap1) is important for attenuating this oxidative stress.

With these data pointing to Nrf2 activity playing a critical role for cell survival during nutrient deprivation-induced autophagy, we then tested whether the antioxidants, N-acetyl cysteine and glutathione, could also protect cell survival during autophagy. Indeed, these two antioxidants protect survival of Nrf2-depleted cells experiencing glucose deprivation (Fig 4.5B), suggesting that the role of increased Nrf2 during glucose deprivation involves coordinating an anti-oxidant response.

4.3.4Induction of the Nrf2 transcription factor is independent of autophagy and oxidative stress

As discussed above, these increases in Nrf2 levels were unexpected in light of the decreased levels of p62 that I observed in glucose-deprived cultures. Therefore, I investigated whether increases in Nrf2 were dependent or independent of autophagy.

Using strategies to block autophagy that were described in the previous chapter, I found that Nrf2 levels increase in glucose-deprived cells, even when cultures are treated with chloroquine (Fig 4.6). Furthermore, increases in Nrf2 occur in glucose-deprived cells with knock-out of ATG or Beclin. Therefore, I conclude that increases in Nrf2 levels are not direct consequence of glucose deprivation- induced autophagy.

Nrf2 has an important role in protecting cells from oxidative stress, and oxidative stress can actually induce increased Nrf2 activity by inactivating KEAP1 (76, 77). To determine whether oxidative stress is important for the induction of Nrf2 in glucose deprivation, I tested whether N-acetyl cysteine affects the decreases in p62 and increases in Nrf2 levels during glucose deprivation. As shown in Figure 4.7, N-acetyl cysteine does not affect the decreases in p62 and increases in Nrf2 levels during glucose deprivation, suggesting that neither of these processes are initiated by high levels of reactive oxygen species. Thus, other mechanisms for induction of Nrf2 are evidently involved in this setting.

4.4 DISCUSSION

The previous chapter described how, using breast cancer cells, I found that glucose deprivation resulted in autophagy, which in turn allowed survival of the cells for approximately three days in starvation conditions. The importance of autophagy in cell survival in these starvation conditions was demonstrated by reduced clonogenicity when autophagy is blocked by pharmacological agents or by knockout of proteins important in autophagy. Autophagy initiated by glucose deprivation also leads to decreases in p62. In light of the previously described relationships between p62, Keap1 and Nrf2, my finding that levels and activity of the antioxidant protein, Nrf2, increase during glucose deprivation was surprising. These increases in Nrf2 protein levels are not dependent on autophagy, since Nrf2 increased in cells with knockout of ATG7. I also explored the possibility that oxidative stress is responsible for increasing levels of Nrf2 during glucose deprivation, and indeed, we did find increased levels of ROS in glucose deprived cultures. This increase in ROS was attenuated by an increase in the level of Nrf2 signaling as evidenced by Keap1 knockdown cells. However, introduction of NAC into glucose-free conditions did not change the increase of Nrf2. Therefore, we conclude that the induction of Nrf2 does not rely on reactive oxygen species. Nrf2 can also be activated by various transcriptional, epigenetic, and post-translational mechanisms and the question of what mechanisms are responsible for activation of Nrf2 in glucose deprivation should be investigated in future studies. In addition, Nrf2 is obviously important for survival of breast cancer cells as demonstrated by the impaired survival of Nrf2 knockdown cells grown in glucose free media. This was confirmed by drug studies in which glucose deprived cells treated with an Nrf2 inhibitor showed decreased survival compared to

untreated glucose deprived controls. This has important implications for treatment of cancer as targeting Nrf2 may be a way to increase tumor killing.

4.5 FIGURES

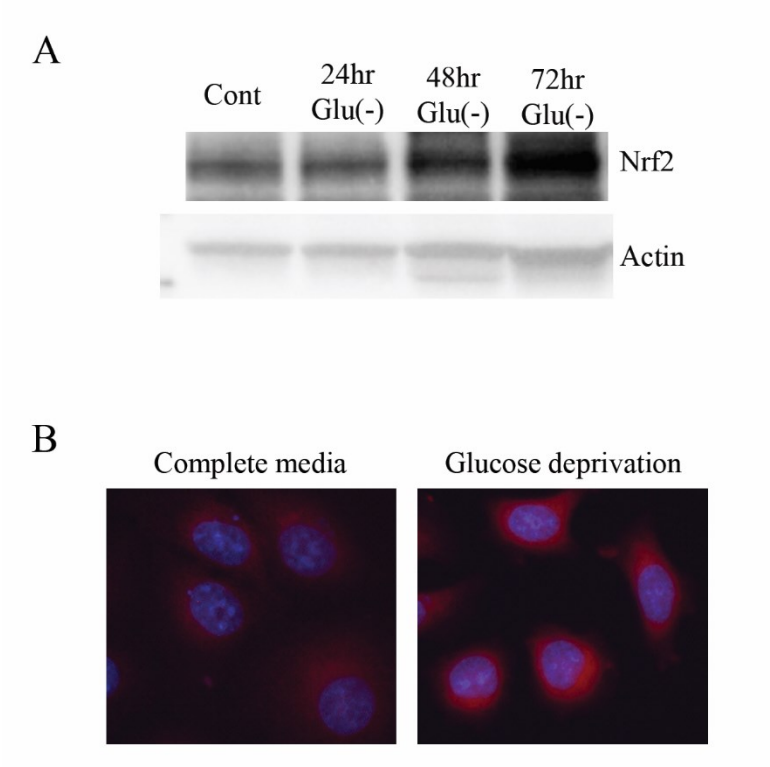

Figure 4.1 Nrf2 is activated by nutrient deprivation. (A) Increased expression of Nrf2 is seen in MCF7 cells grown in glucose free media for 24, 48, and 72 hours. (B) Increased expression of NQO1, an Nrf2-regulated transcript, in MCF7 cells after 48 hours of glucose deprivation. Fluorescence immunohistochemistry was used to measure Nrf2 levels in MCF7 cultures grown in complete media or glucose-free media for 48 hours. (red= NQO1, blue=DAPI)

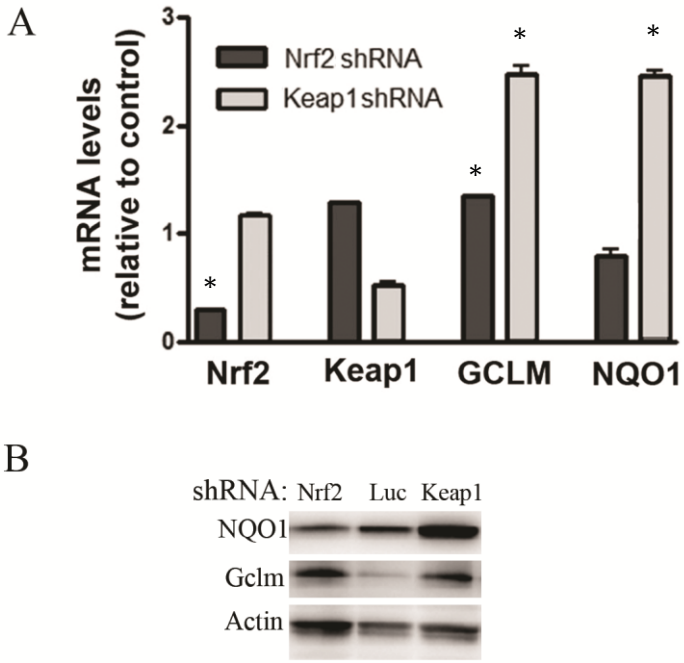

Figure 4.2 Knockdown of Keap1 and Nrf2 in MCF7 cells (A) After transfection of MCF7 cells with Nrf2 shRNA , Keap1 shRNA, or control (luciferase) shRNA, mRNA levels of Nrf2, Keap1, Gclm, and NQO1 were measured using q-PCR. Differences compared to control that are significant by t-test are designated by asterisks. (p=0.0039 Nrf2 mRNA: LucshRNA vs. Nrf2shRNA, p=0.0323 Gclm mRNA: LucshRNA vs. Nrf2shRNA, p=0.005 Gclm mRNA: LucshRNA vs. Keap1shRNA, p=0.003 NQO1 mRNA: LucshRNA vs. Keap1shRNA) (B) The effect of Nrf2 and Keap1 knockdown on Nrf2's downstream target antioxidant proteins, Gclm and NQO1, were also observed by immunoblotting. Both proteins are increased, as expected, when Keap1 is knocked down thus activating Nrf2.

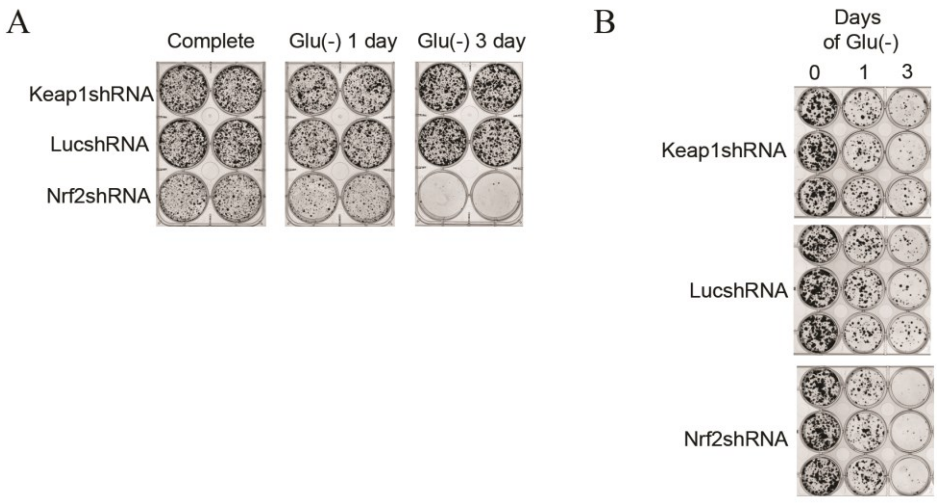

Figure 4.3 Nrf2 is important in the survival of MCF7 cells during glucose deprivation. (A & B)MCF7shRNA cells were cultured in complete or glucose free media for 1 or 3 days. Cells were replenished with complete media and allowed to grow until colonies are visible. Nrf2shRNA cells have impaired survival during glucose deprivation when compared to cells with intact Nrf2 signaling.

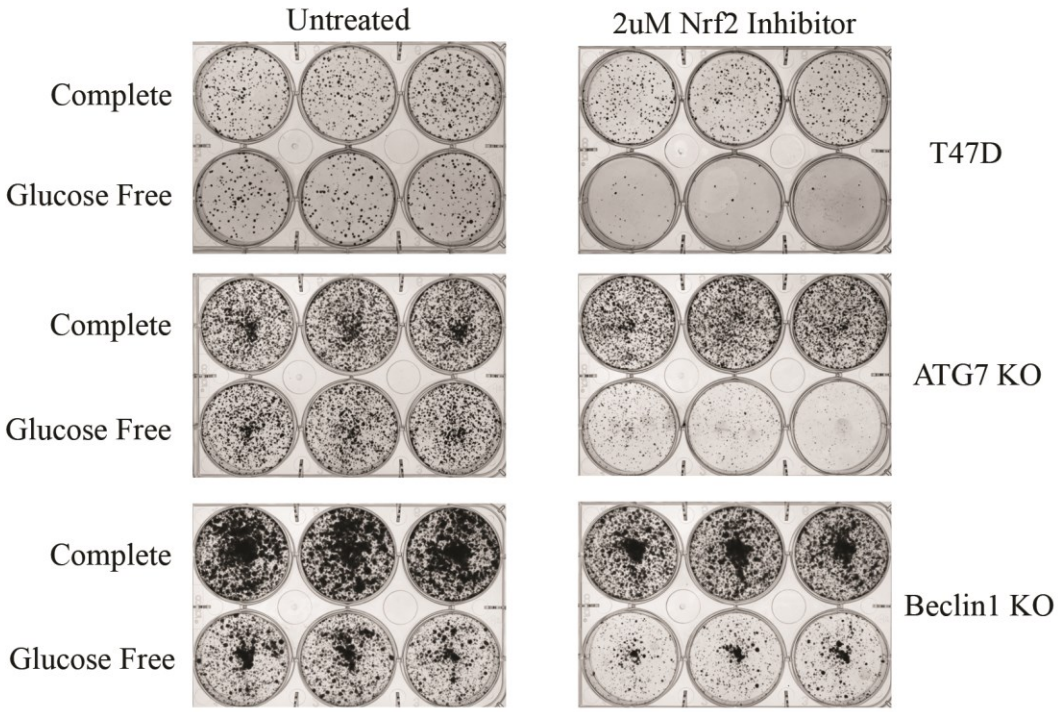

Figure 4.4 Both autophagy competent and autophagy deficient cells have decreased survival when treated with an Nrf2 inhibitor. Cells were grown in complete or glucose free media with or without 2uM of the Nrf2 Inhibitor, ML-385, for 3 days. After 3 days, cells were replenished with complete media with no drug and allowed to grow until colonies were stained with crystal violet.

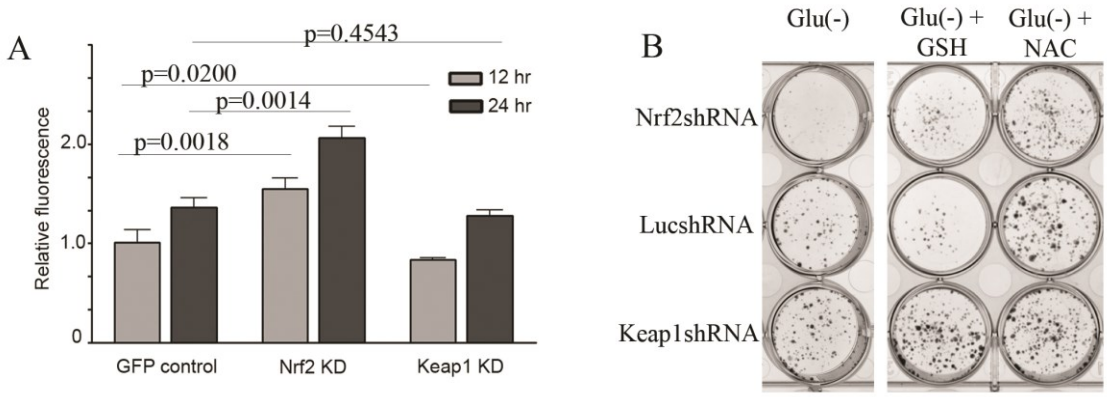

Figure 4.5 Nrf2 regulates protection from reactive oxygen species (ROS) produced during autophagy. (A) Time-dependent increases in levels of ROS during glucose deprivation, as measured using the DCFDA fluorescent probe. When compared to control cultures (GFP shRNA), Keap1 knock-down resulted in modestly decreased levels of ROS as measured after 12 and 24 hours of glucose deprivation (p=0.02 at 12 hr, p=0.45 at 24 hr). Nrf2 knock down resulted in significantly higher levels of ROS after glucose deprivation (p=0.0018 at 12 hr, p=0.0014 at 24 hr). (B) Incubation of cultures with glutathione (GSH) or N-acetyl cysteine (NAC) increases cell survival during glucose deprivation, especially in cells with knock-down of Nrf2. All cultures were incubated for 3 days in glucose free media with or without antioxidants, followed by incubation in complete media for an additional 10 days to allow colony growth from surviving cells.

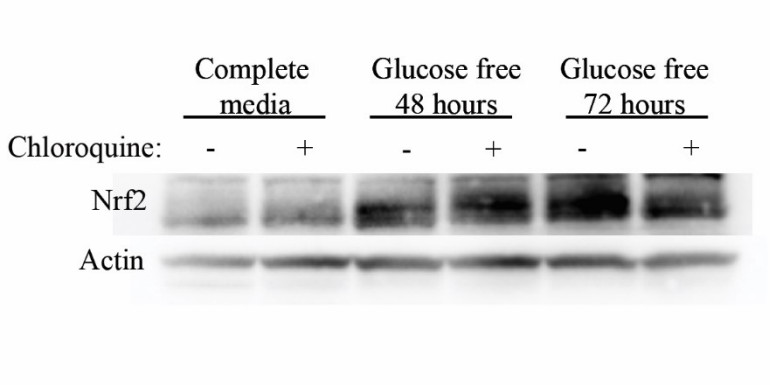

Figure 4.6 Nrf2 is upregulated despite blocking the completion of autophagy. Cells were grown in complete or glucose free media for 48 or 72 hours with or without 25uM chloroquine. Nrf2 was assayed by immunoblot.

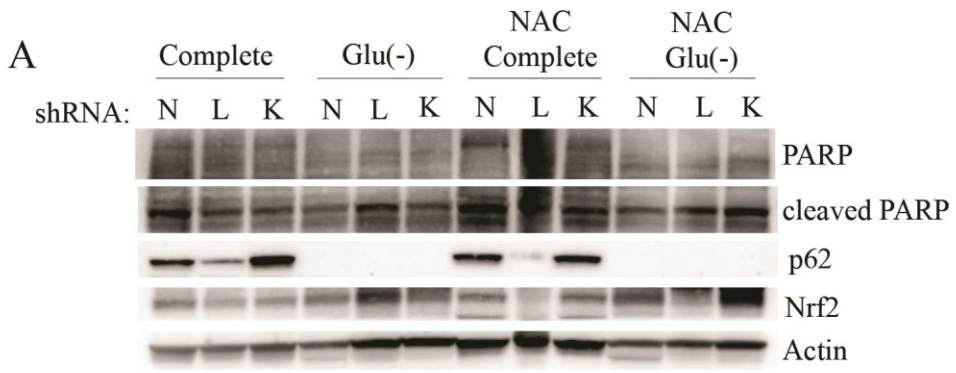

Figure 4.7 Supplementing glucose free media with NAC does not prevent decreases in p62 or upregulation of Nrf2. Cells were grown in glucose free or complete media with or without 2mM NAC for 48 hours. P62 and Nrf2 were assayed by immunoblot. (N=MCF7 Nrf2shRNA, L= MCF7 LucshRNA, K= MCF7 Keap1shRNA)

Chapter 5 Effect of hypoxia on Nrf2 signaling and survival of breast cancer cells

5.1 INTRODUCTION

Hypoxia is another common stressor that cancer cells encounter and usually in the same areas as glucose deprivation would occur due to poor vasculature (51). As discussed in chapter 4, Nrf2 is important for survival of breast cancer cells during glucose deprivation so hypoxia was considered as another stressor in which Nrf2 might have a role. Furthermore, there is some evidence as discussed by Chun et al. that reactive oxygen species may affect the stabilization of HIF1α and as presented in background, Nrf2 is relevant in the antioxidant response. The data on ROS and stabilization of HIF1α is inconclusive as some data show HIF1α stabilization with decreased ROS whereas other studies show increased ROS cause HIF1α stabilization (78-81).

In this chapter I'll describe how breast cancer cells survive hypoxic conditions and that Nrf2 plays a role in the survival of hypoxic cells. Hypoxia in cells grown in complete media show increased antioxidant protein expression compared to normoxia. However, when glucose deprivation and hypoxia are combined, autophagy and Nrf2 signaling is attenuated. These studies are not all encompassing but provide preliminary data for future studies.

5.2 MATERIALS AND METHODS

5.2.1 Cell Culture

Human cancer cell line MCF7 was cultured in DMEM media (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). T47D was cultured in RPMI (Gibco) supplemented with 10% FBS. Experiments requiring glucose deprivation used RPMI glucose free media (Gibco) 0.1%FBS. DMEM glucose free media was made from DME base (Sigma Aldrich) dissolved in ultrapure water and supplemented with sodium bicarbonate, sodium pyruvate, L-glutamine and 0.1%FBS. MCF7shRNA cells were maintained in DMEM with 750ng/ml of puromycin.

5.2.2 Reagents

Primary antibodies for NQO1 were purchased from Cell Signaling Technology. LC3B and GCLM were purchased from Novus Biologicals. HIF1α and Nrf2 antibodies were purchased from Santa Cruz. Anti-Actin antibody was purchased from Sigma-Aldrich. Anti-rabbit and anti-mouse secondary antibodies were purchased from Cell Signaling Technology. Puromycin was purchased from Sigma and dissolved in ultrapure water to make a 1mg/ml stock solution.

5.2.3 Clonogenic Assays

MCF7 cells were plated at appropriate density ranging from 500 to 3000 cells per well. After 24 hours incubation, cells were treated with complete or glucose free medium for variable amounts of time with the most common being 72 hours. Cells were incubated in

either normoxic or hypoxic (1% oxygen) conditions. After treatment with the different mediums and oxygen levels, cells were replenished with complete media without drug and allowed to grow in normoxic conditions for an average time of 10 days before being fixed with 100% methanol and stained with 0.5% crystal violet (Sigma).

5.2.4 Immunoblot

After experimental treatment, cells were harvested in TNE lysis buffer plus protease inhibitor and protein concentration was determined using Pierce BCA assay (Thermo Fisher Scientific). Equal amounts of protein were loaded for each sample, separated by SDS-PAGE on a 10% or 10-20% gel Tris-HCl gel (Bio-Rad) and incubated overnight with primary antibody at 4ºC. Most antibodies were incubated at a 1:1000 concentration. Exceptions include actin (1:20,000), and sigma antibodies (1:500). After washing and a 1.5 hour incubation with anti-rabbit or anti-mouse secondary antibody at room temperature, membranes were developed with SuperSignal West Femto Max Sensitivity Substrate(Thermo Fisher Scientific). Some membranes were stripped with Stripping Buffer (Thermo Fisher Scientific) and re-probed with other primary antibodies.

5.2.5 Hypoxia

The hypoxic atmosphere was produced by filling a glove box (Plas Labs Basic Glove Box 818-GB) with nitrogen until the atmosphere reached 1% oxygen as indicated by the gas sensor, Drager Pac 7000. A small incubator was put in the glove box and humidity was introduced using a humidifier. All hypoxic experiments were carried out at 1% oxygen.

5.3 RESULTS

5.3.1 Breast cancer cells are capable of surviving hypoxic conditions.

As discussed above, breast cancer cells are able to survive glucose deprivation for approximately 3 days. Tumors exhibit areas of both nutrient deprivation and hypoxia and these factors may be important in how a cancer cell acts in the tumor microenvironment and reacts to drug. Therefore, I decided to study how hypoxia affects breast cancer cells. Figure 5.1 shows cells cultured in glucose free media and hypoxia and then replenished with complete media are able to proliferate. Establishing cell viability during hypoxia led to further inquiries into the behavior of cells with different levels of Nrf2 signaling.

5.3.2 Nrf2 is important in determining survival of breast cancer cells in hypoxia.

Nrf2 was shown to be important in survival of breast cancer cells during glucose deprivation in chapter 4. In addition to studying Nrf2 signaling in glucose deprivation, I wanted to study its effect during hypoxia. When subjected to hypoxia alone, there is less cell growth than in normoxic conditions and as in glucose deprivation, Nrf2shRNA cells had impaired survival compared to control. Surprisingly, in Figure 5.2, hypoxia has no additive effect on cell death when cells are grown in glucose free media. Survival is again poorest for Nrf2shRNA cells but in all shRNA cases, hypoxia does not increase the amount of cell killing in cells grown in glucose deprived conditions.

5.3.3 Hypoxia attenuates the response to glucose deprivation

As seen in chapter 3 and 4, glucose deprivation induces autophagy and increases Nrf2 signaling. Figure 5.3 shows similar results with increased LC3II and Gclm when

MCF7shRNA are cultured in glucose free conditions. Compared to complete media, normoxic conditions, in the complete media, hypoxic setting, autophagy seems to be unchanged while Nrf2 signaling may be increased (Fig 5.3). When combining the conditions of glucose deprivation and hypoxia, autophagy is attenuated and there's a decrease in Nrf2 signaling. Interestingly HIF1α expression is also attenuated when cells are cultured in glucose deprivation and grown in hypoxic conditions.

5.4 DISCUSSION

As with glucose deprivation, hypoxia is a metabolic stressor that cancer cells encounter within their tumor microenvironment. I was able to show that breast cancer cells are able to resume proliferation after a hypoxic stressor. While Nrf2shRNA was expected to have the most impaired survival in hypoxic conditions, it was unexpected that hypoxia and glucose deprivation do not have a synergistic effect on cell death. It seems that the predominate stressor, glucose deprivation, dictates the survival and death pathways that are activated and/or inhibited. This is corroborated by the finding that HIF1α expression was attenuated in the hypoxia/glucose deprivation combination growth conditions. According to several studies, this decrease in HIF1α might be due to decreases in ATP levels (52-54). Because glucose deprivation and hypoxia occur in the same areas of the tumor microenvironment, it is crucial to study their effects on cancer cell survival and their pathways in order to target the tumor cells more effectively.

5.5 FIGURES

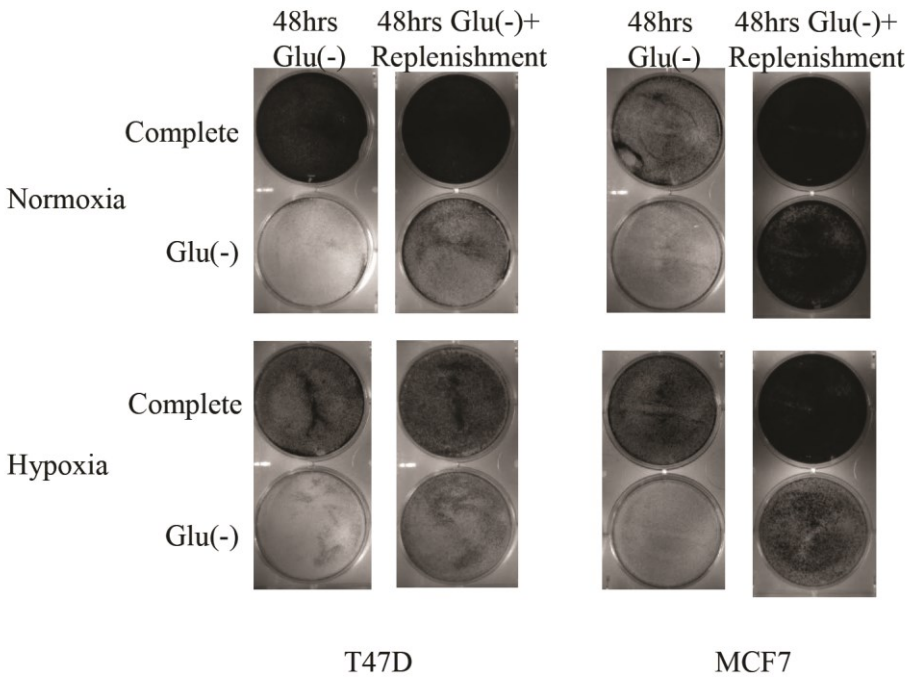

Figure 5.1 Cells are able to survive hypoxic conditions. MCF7 and T47D cells were culture in complete or glucose free media and in normoxic or hypoxic (1% oxygen) conditions for 48 hours. Cells were then stained with crystal violet or replenished with complete media and place into a normoxic incubator until colony growth and staining.

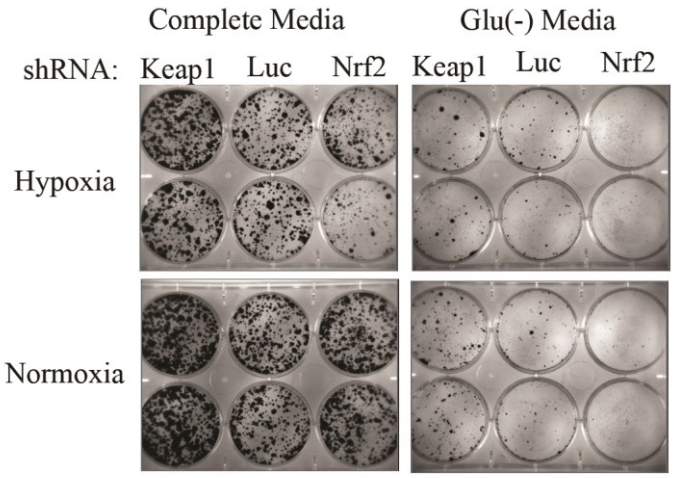

Figure 5.2 Nrf2 is important in cell survival during hypoxia. MCF7shRNA cells were cultured in complete or glucose free media and placed in either a normoxic or hypoxic (1% oxygen) incubator for 72 hours. Cells were then replenished with complete media and allowed to grow in normoxic conditions until colonies were visible at which time, plates were stained with crystal violet.

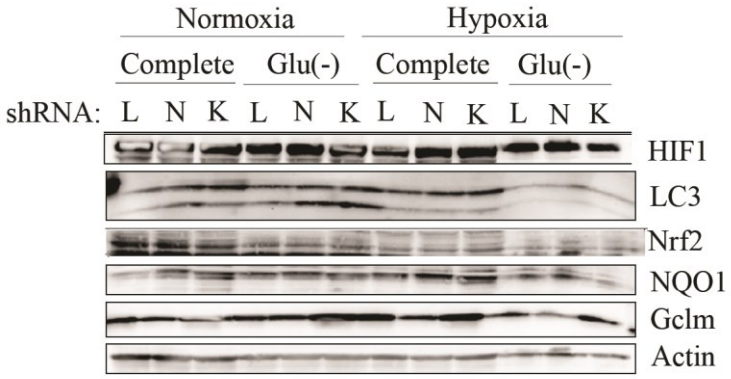

Figure 5.3 Hypoxia attenuates the cell's response to glucose deprivation.

MCF7shRNA cells were cultured in complete or glucose free media in a normoxic or hypoxic (1% oxygen) incubator for 2 days. The following proteins were assessed by immunoblot: HIF1α, LC3, Nrf2, NQO1, and Gclm. (L= MCF7 lucshRNA, N= MCF7 Nrf2shRNA, K= MCF7 Keap1shRNA)

Chapter 6 Hypoxia and Glucose Deprivation affect Expression of ER and HER2 in Breast Cancer Cells

6.1 INTRODUCTION

Early in my graduate career, during a lab meeting we were discussing the topics of HER2 positive breast cancers and autophagy when an idea came to me that perhaps autophagy, a process that degrades macromolecules and proteins, might be degrading human epidermal growth factor receptor 2 and estrogen receptor of HER2 and ER positive cancer cells causing resistance to targeted drug therapies. At that time I had no preliminary data or knowledge to back up this hypothesis. With further research into drug resistance and autophagy, there was a possibility that this hypothesis may have a basis to it.

Qadir Ma et al conducted a study in which autophagy was found to be increased in cells undergoing treatment with tamoxifen and that inhibition of autophagy could sensitize cells to the drug (59). Another investigator found that HER2 levels were decreased in trastuzumab resistant cells when maintained in drug (62).

As it turns out and you will see described in this chapter, receptors of these two subtypes of breast cancer are downregulated during glucose deprivation. However, autophagy does not seem to be the mechanism of down-regulation. Additional studies were performed to determine the mechanism of downregulation along with experiments aimed at determining the efficacy of targeted drugs in the glucose deprived cells. Interestingly, despite receptor downregulation, targeted drugs were effective in the glucose deprivation setting and even more so than cells grown in complete media with intact receptors.

6.2 MATERIALS AND METHODS

6.2.1 Cell Culture

Human cancer cell lines MCF7 and BT474 were cultured in DMEM media (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco). T47D and SKBR3 were cultured in RPMI (Gibco) supplemented with 10% FBS. Experiments requiring glucose deprivation used RPMI glucose free media (Gibco) 0.1%FBS. DMEM glucose free media was made from DME base (Sigma Aldrich) dissolved in ultrapure water and supplemented with sodium bicarbonate, sodium pyruvate, L-glutamine and 0.1%FBS.

6.2.2 Reagents

Primary antibodies for HER2 were purchased from Cell Signaling Technology. HIF1α and ER (estrogen receptor) antibodies were purchased from Santa Cruz. Anti-Actin antibody was purchased from Sigma-Aldrich. Anti-rabbit and anti-mouse secondary antibodies were purchased from Cell Signaling Technology. Chloroquine, a pharmacological inhibitor of autophagy, was dissolved in water to make a 25mM stock concentration. Rapamycin was diluted in water to make a 27uM stock solution. Lapatinib was purchased from LC Laboratories and dissolved in DMSO for a 200mg/ml stock solution. Trastuzumab/Herceptin was kindly provided by Genetech and a 1mg/ml stock solution was made. 4-hydroxytamoxifen was purchased from Sigma and dissolved in ethanol to make a 5mM solution. Tamoxifen was purchased from Sigma and dissolved in DMSO to make a 10mM stock solution. Bortezomib was dissolved in DMSO for a 1mM stock solution.

6.2.3 Clonogenic Assays

MCF7 and T47D were plated at appropriate density ranging from 500 to 3000 cells per well. After 24 hours incubation, cells were treated with complete or glucose free medium for variable amounts of time with the most common being 72 hours. In other experiments, cells were treated with complete or glucose free media long with variable concentrations of chloroquine, 1uM 4OHT, 1uM tamoxifen, 1mM lapatinib, or 1ug/ml trastuzumab according to the experiment. After treatment with the different mediums and drugs, cells were replenished with complete media without drug and allowed to grow for an average time of 10 days before being fixed with 100% methanol and stained with 0.5% crystal violet (Sigma).

6.2.4 Immunoblot

After experimental treatment, cells were harvested in TNE lysis buffer plus protease inhibitor and protein concentration was determined using Pierce BCA assay (Thermo Fisher Scientific). Equal amounts of protein were loaded for each sample, separated by SDS-PAGE on a 10% or 10-20% gel Tris-HCl gel (Bio-Rad) and incubated overnight with primary antibody at 4ºC. Most antibodies were incubated at a 1:1000 concentration. Exceptions include actin (1:20,000), and sigma antibodies (1:500). After washing and a 1.5 hour incubation with anti-rabbit or anti-mouse secondary antibody at room temperature, membranes were developed with SuperSignal West Femto Max Sensitivity Substrate(Thermo Fisher Scientific). Some membranes were stripped with Stripping Buffer (Thermo Fisher Scientific) and re-probed with other primary antibodies.

6.2.5 RT-PCR

Cells were harvested after 48 hours of growth in complete or glucose free media and RNA isolated with RNeasy Mini Kit (Qiagen) and eluted in water. RNA quality and concentration were measured by Nanodrop ND-1000 Spectrophotometer. The reverse transcription reaction was on a ThermoHybaid. The PCR reaction was performed on a ThermoHybaid. Samples were then run on a 1.3% agarose gel in TBE buffer and visualized using UV on a BioRad ChemiDoc System.

Pre-RT mixture	7ul
RNaisn (28U/ul)	0.5ul
MMLV (200U/ul)	0.5ul
Total RNA (3.5ug) + DEPC-water	12ul
	Total Volume= 20ul

Table 6.2.1 Reverse transcription/cDNA synthesis reaction mixture

5X RT buffer	400 ul
100mM dATP	5ul
100mM dTTP	5ul
100mM dGTP	5ul
100mM dCTP	5ul
50pM Oligo d(T)	100ul
DEPC-water	180ul
	Total Volume= 700ul

Table 6.2.2 Pre-RT mixture for cDNA synthesis

42ºC	60 minutes
94ºC	5 minutes

Table 6.2.3 Reverse transcription cycling conditions

5X GoGreen Buffer (Promega	10ul
Sense primer	0.25ul
Antisense primer	0.25ul
GoTaq Flexi DNA Polymerase (Promega)	0.25ul
cDNA	3ul
25mM MgCl2	5ul
10mM dNTP	1ul
DMSO	2.5ul
dH20	27ul
	Total Volume= 50ul

Table 6.2.4 PCR reaction mixture

1 cycle	94ºC for 1 minute
23 cycles	94ºC for 30 seconds, 58ºC for 30 seconds, 72ºC for 30 seconds
1 cycle	72ºC for 5 minutes
Hold	4ºC

Table 6.2.5 PCR reaction cycling conditions

GENE	REFERENCE SEQUENCE	OLIGONUCELOTIDE SEQUENCE
Estrogen Receptor	NM_000125.3	Forward: 5'CCAGGGAAGCTACTGTTTGCTC3'
		Reverse: 5'GCTGTACAGATGCTCCATGCC3'

Table 6.2.6 Oligonucleotide sequence for reverse transcriptase PCR

6.2.6 Hypoxia

The hypoxic atmosphere was produced by filling a glove box (Plas Labs Basic Glove Box 818-GB) with nitrogen until the atmosphere reached 1% oxygen as indicated by the gas sensor, Drager Pac 7000. A small incubator was put in the glove box and humidity was introduced using a humidifier. All hypoxic experiments were carried out at 1% oxygen.

6.3 RESULTS

6.3.1 Breast Cancer Cells are Viable after 48 hours of glucose deprivation.

Tumors are subject to areas of ischemia which include hypoxia and nutrient deprivation. To determine whether cancer cells are able to survive this stress, breast cancer cell lines were cultured in glucose free media for 48 hours and replenished with full media. As seen in Figure 6.1, cell lines SKBR3, BT474, MCF7, and T47D are able to survive and retain the ability to proliferate when nutrients are plentiful.

6.3.2 Receptors are down-regulated in response to glucose deprivation.

Resistance to targeted drug therapies is an important concept to study. Since its been shown that resistant cells benefit from inhibiting autophagy and that receptors in drug resistant cells have decreased growth factor receptors (59, 62), I decided to study the effect of glucose deprivation on the drug target of HER2 and ER + cell lines. Surprisingly, both HER2+ and ER+ cell lines lose expression of HER2 and ER respectively during glucose deprivation and this loss is apparent even at 120 hours (Fig 6.2).

6.3.3 Response of breast cancer receptors to hypoxia.

In addition to glucose deprivation occurring in tumors, so does hypoxia. Therefore, I decided to study the effect of hypoxia on the receptors of breast cancer to determine if it followed the discovery made during glucose deprivation. Cells cultured in complete media and hypoxia upregulate the protein HIF1α as expected. Unexpectedly, HER2 and ER are upregulated in hypoxic conditions compared to normoxia (Fig 6.3A). Another unanticipated observation was made when breast cancer cells were cultured in

glucose deprivation and hypoxic conditions. Glucose deprivation attenuated the HIF1α response while maintaining the down-regulation of receptors (Fig 6.3B)

6.3.4 Autophagy, the proteasome, and decreased transcription as potential mechanisms of receptor downregulation

Both autophagy and the proteasome are involved in degradation of proteins. Autophagy was studied first since the downregulation of receptors was occurring in a metabolic stress situation. Rapamycin induced autophagy showed no decrease in the estrogen receptor (Fig 6.4A). Time and dose response to rapamycin was also studied to ensure that estrogen receptor was not being degraded (Fig 6.4A and Fig 6.4B). Autophagy induced by glucose free media was also shown to not affect receptor levels. As seen in Figure 6.5 autophagy is induced, evidenced by the increase in LC3II in both glucose free cells and glucose free cells that have been replenished with complete media. However, estrogen receptor is present in the replenishment condition even though LC3II is present and autophagy is occurring. Also, chloroquine was used to block autophagy and as can be seen in the 48 hour glucose free conditions with chloroquine (Fig 6.5), estrogen receptor is still downregulated. Therefore autophagy could not be the mechanism of down-regulation. Because autophagy was ruled out as the mechanism of receptor downregulation, the proteasome was investigated next. Inhibition of the proteasome did not result in increased levels of HER2 or ER, therefore I concluded that proteasome degradation was unlikely to be the reason for receptor downregulation in glucose free conditions (Fig 6.6). A final mechanism of ER downregulation was assessed- mRNA expression of estrogen receptor was studied. As seen in Figure 6.7, glucose free media had low mRNA levels of estrogen receptor when compared to

complete media. This preliminary finding that reduced transcription may be the cause of receptor downregulation should be followed up.

6.3.5 Drug efficacy of breast cancer cells during glucose deprivation.

In addition to studying the mechanism of receptor downregulation in glucose deprivation, drug studies were performed to test their efficacy in glucose free media. Presumably a down regulation of the drug target would render the cell resistant. In fact, the opposite was discovered. Cells grown in glucose free media treated with drug actually had worse survival than cells grown in complete media and treated with drug (Fig 6.8). This is totally unexpected as discussed above, cells in glucose free media no longer have receptors whereas those in grown in complete media do. It should also be noted that estrogen receptor positive cells grown in complete media and treated with the HER2 drug, trastuzumab, had significantly decreased survival even in comparison to the tamoxifen treated wells (Fig 6.9). And in another surprising result, this decrease in survival was even greater in complete media than in glucose free media (Fig 6.9).

6.4 DISCUSSION

The treatment of HER2 positive and ER positive breast cancers involves drugs targeted to growth factor receptors. The efficacy of such drugs is important to understand in the context of glucose deprivation as it's a microenvironmental stressor present in tumors. In our studies, the receptors of the ER and HER2 positive breast cancer cell lines are downregulated during glucose deprivation. However, despite autophagy being induced in the glucose deprivation setting, it does not seem to be the mechanism of downregulation. Proteasome degradation was also found to be an unlikely mechanism. Preliminary studies on mRNA levels of estrogen receptor found that ER mRNA was decreased in glucose deprivation and this possible aspect of down regulation needs to be studied further. Knowing that receptors were downregulated in glucose deprivation, I was interested in seeing its effect on the efficacy of the targeted drugs to HER2 positive and ER positive cell lines. Quite interestingly drugs targeting ER and HER2 had more cell killing in the glucose deprivation setting compared to complete media. But without receptors to act on, how is this drug working? Tamoxifen is known to stabilize estrogen receptor (82) so perhaps tamoxifen also stabilizes receptors in glucose deprivation thus allowing killing of cells. A colleague suggested another idea that this decrease in receptors may be due to increased turnover, indicating increased activity of estrogen receptor, and thus blocking activity with tamoxifen would be catastrophic to these cells. More work needs to be done in order to determine how the drug is acting to kill cells without its target. Trastuzumab, a drug approved for breast cancer overexpressing HER2, has cell killing effects in ER positive breast cancer cells grown in complete media. Not only is the drug effective in that setting, but is less effective in ER positive breast cancer

cells grown in glucose free media. The reason for this result also needs to be followed up. The beginning of this story of estrogen receptor and human epidermal growth factor receptor is a curious one with the possibility to impact clinical treatment of breast cancer.

6.5 FIGURES

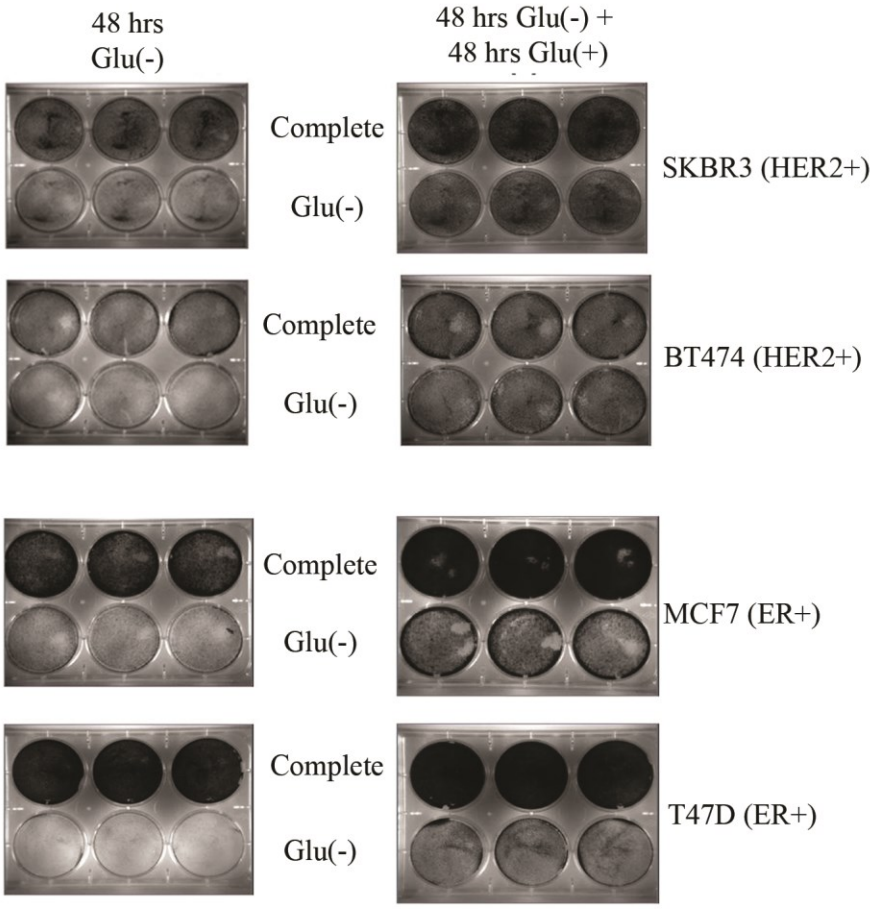

Figure 6.1 Breast cancer cells are viable after 48 hours of glucose deprivation.

HER2+ and ER+ cell lines were cultured in glucose free media for 48 hours and stained. A second set of plates were stained after being replenished with complete media after the 48 hour glucose deprivation period.

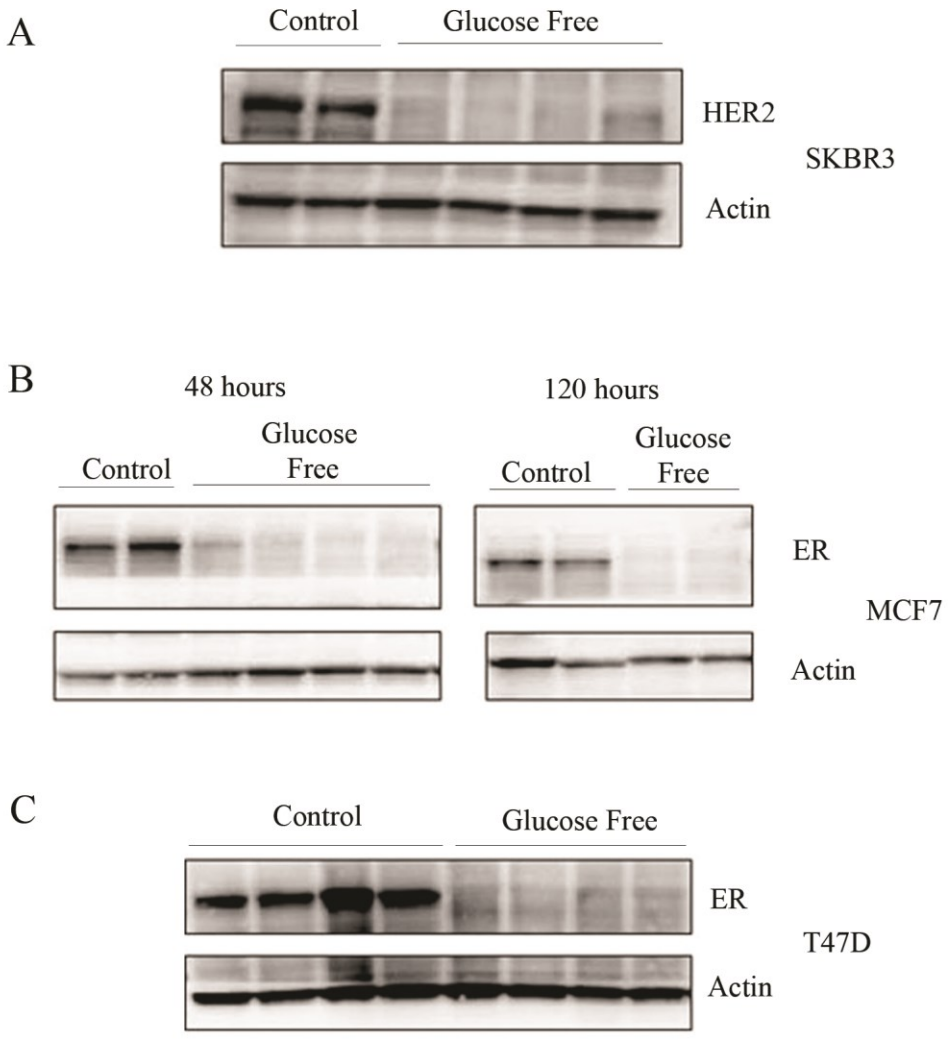

Figure 6.2 Receptors in HER2+ and ER+ cell lines are down-regulated in response to glucose deprivation. (A) SKBR3 cells were cultured in complete or glucose free media for 48 hours. Immunoblot was performed for HER2. (B) MCF7 cells were cultured in complete or glucose free media for 48 and 120 hours. Estrogen receptor was assayed by immunoblot. (C) T47D was grown in complete or glucose free media for 48 hours. Estrogen receptor was visualized by immunoblot.

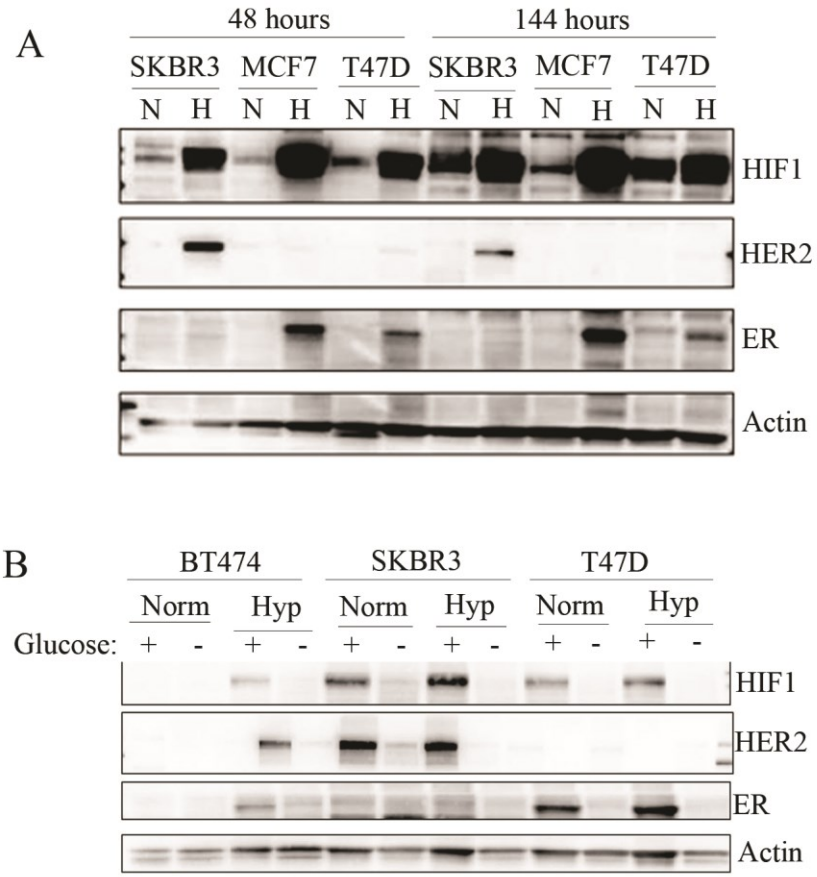

Figure 6.3 Breast cancer cells in a hypoxic environment upregulate their receptors while glucose deprivation prevents HIF1α upregulation. (A) Cells were grown in complete media in either normoxic or hypoxic(1% oxygen) conditions. Cells were harvested at 48 and 144 hours. HIF1α and receptors were assayed by immunoblot. (B) Cells were cultured in complete or glucose free media in either normoxic or hypoxic conditions. Cells were harvested 48 hours later and assayed for HIF1α, HER2, and ER by immunoblot. N/Norm= normoxia, H/Hyp= hypoxia

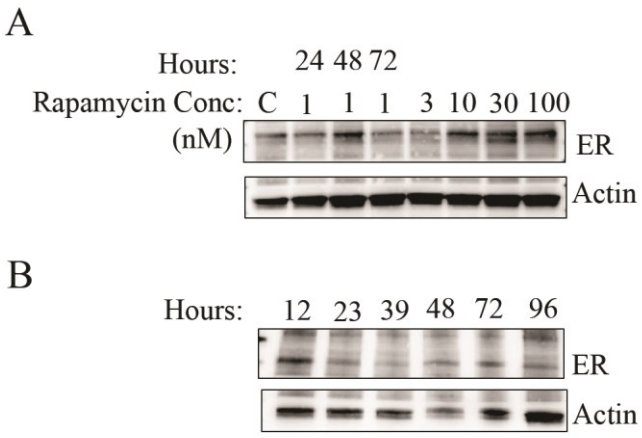

Figure 6.4 Rapamycin induced autophagy does not reduce the expression level of estrogen receptor in T47D cells. (A) T47D cells were cultured in complete media and treated with increasing concentrations of rapamycin (1, 3, 10, 30, 100nM). At the 1nM concentration cells were treated for 24, 48, or 72. All other rapamycin concentrations were harvested at the 24 hour timepoint. ER was assayed by immunoblot. (B) T47D cells were grown in complete media and treated with 1nM rapamycin and harvested at varying timepoints. Cells were assayed for ER by immunoblot.

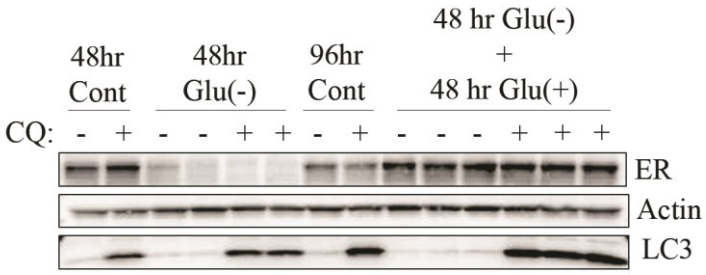

Figure 6.5 Glucose deprivation induced autophagy does not downregulate estrogen receptor. MCF7 cells were grown in complete or glucose free media with or without 25uM chloroquine for 48 or 96 hours. Cells grown in glucose free media for 48 hours were replenished with complete media for an additional 48 hours. Estrogen receptor and LC3 were visualized by immunoblot. CQ= chloroquine

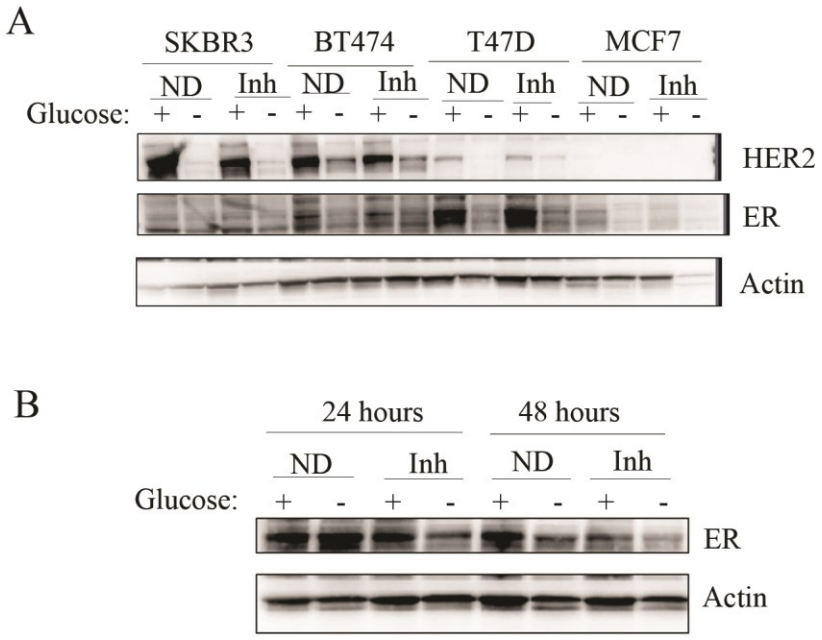

Figure 6.6 Proteasome not responsible for the degradation of ER and HER2. (A) Breast cancer cell lines were grown in complete or glucose free media for 2 days. Bortezomib (1uM) was added to appropriate plates 4 hours before harvesting cells. HER2 and ER were visualized by immunoblot. (B) T47D cells were grown in complete or glucose free media for 24 and 48 hours. Bortezomib (1.25 uM) was added 4 hours before harvesting. ER was assessed by immunoblot. ND= no drug, Inh= proteasome inhibitor Bortezomib

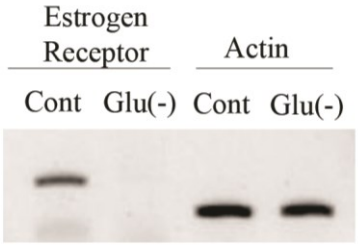

Figure 6.7 Transcription of estrogen receptor is reduced during glucose deprivation. T47D cells were grown in complete or glucose free media for 2 days. Reverse transcriptase-PCR was performed to measure mRNA levels of the transcript estrogen receptor.

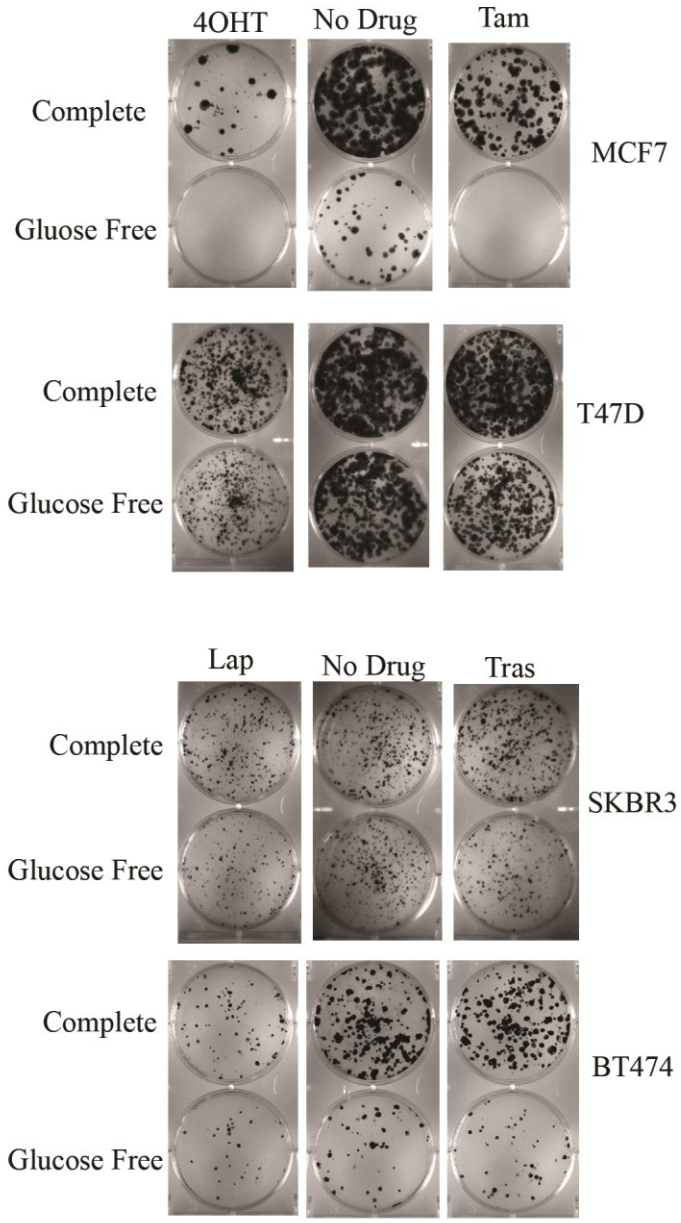

Figure 6.8 Breast cancer cells grown in glucose free media are more sensitive to targeted therapies than those grown in complete media. Cells were grown in complete or glucose free media with or without 1uM 4-hydroxytamoxifen (4OHT), 1uM tamoxifen (Tam), 1mM lapatinib (Lap), or 1ug/ml trastuzumab (Tras) for 3 days and then replenished with complete media until colonies were visible and stained with crystal violet.

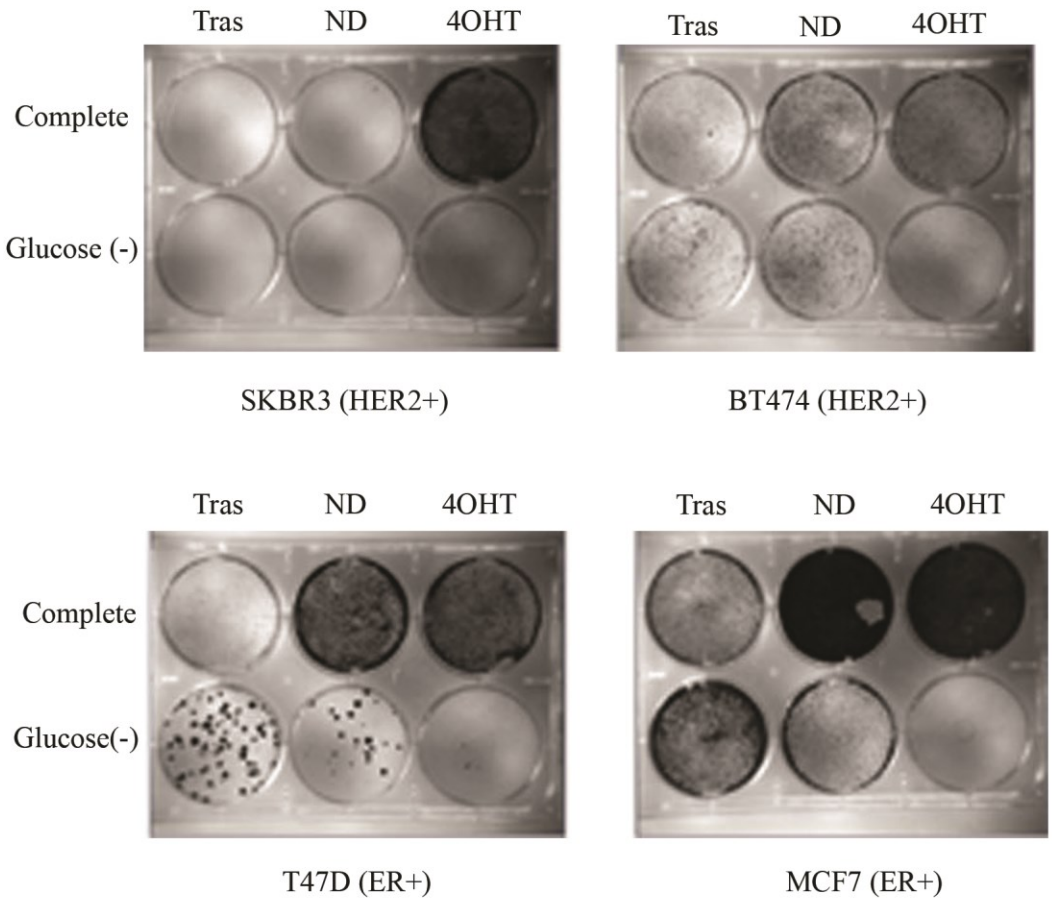

Figure 6.9 Targeted drug therapies still have a killing effect despite absence of receptor. Breast cancer cells were grown in complete or glucose free media and treated with 1ug/ml of Trastuzumab (Tras) or 1uM of 4-hydroxytamoxifen (4OHT) for 3 days. Cells were replenished with complete media, allowed to grow, and then stained with crystal violet. ND=no drug

Chapter 7 Conclusions and Future Directions

In our initial experiments, we found that cultured breast cancer cells (MCF7 and T47D cell lines) are capable of survival for approximately three days in the absence of glucose. Glucose deprivation is common in ischemia, and we found that glucose deprivation results in an induction of autophagy, as evidenced by an increased LC3II: LC3 I ratio and punctate staining for LC3 II, which represent common markers of autophagy (83, 84). Significantly, this autophagy response is critical for survival of these cells during glucose deprivation, and blocking autophagy with pharmacological agents such as chloroquine, or by knock-down of the essential autophagy genes ATG7 or Beclin1, results in rapid death by apoptosis when cells are cultured in glucose-free medium.

Previous studies have not definitively defined the role of autophagy in glucose deprivation (64, 70, 85-87). Several studies reported that knockdown of genes essential for autophagy in cells deprived of serum or amino acids was found to lead to cell death by apoptosis(65, 66), and autophagy has also been shown to protect cells from 2-deoxyglucose-induced cell death (68, 69, 88). While incubating cells with 2-deoxyglucose might be expected to mimic the situation of glucose deprivation, experiments testing glucose-free medium in a variety of cultured cell lines, found no protective role for autophagy in the setting of glucose deprivation(70).

The apparent discrepancy between these results from previous studies on glucose deprivation and the experiments reported here cannot be readily explained. As detailed below, we did find that another survival pathway, activation of Nrf2, is also critical for survival of breast cancer cells deprived of glucose, and it is possible that previous

experiments used cells or conditions that did not result in activation of Nrf2. Another possible explanation for discrepant results could be a result of some cancers being highly dependent on glutamine as a source of energy as well as biosynthetic substrates (89, 90), and for these cells, glucose deprivation might have different consequences on cell survival.

The induction of autophagy and the protective effects of this process during glucose deprivation could be important for survival of cancer cells in situations of metabolic stress, such as ischemia. In fact, substantial data points to autophagy contributing to cancer development in a variety of experimental settings(91). Particularly in cancers with RAS mutations, experimental data indicates that autophagy is critical for growth and survival of cancer cells (92-95). In most cancerous tumors, the disordered microenvironment is associated with metabolic stress due to variable hypoxia and nutrient insufficiency, and in this setting autophagy appears to have an essential role in recycling intracellular macromolecules and organelles to transiently provide essential metabolic substrates in such settings(3). Thus, our observations are consistent with breast cancer cells inducing autophagy as a protective response to glucose deprivation.

I then investigated what protective molecular pathways might be triggered by autophagy in the setting of glucose deprivation. In particular, my studies focused on p62, an LC3-interacting, ubiquitin-associated protein. In various disease settings, p62 has been found to accumulate in cytosolic protein aggregates and in cellular inclusion bodies together with polyubiquitinated proteins. Autolysosomes containing p62- and LC3-positive bodies are degraded by autophagy(74) , resulting in decreased levels of p62.

Interestingly, p62 has been shown to sequester and facilitate degradation of Keap-1 protein, and when autolysosomes containing p62- and LC3-positive bodies are degraded by autophagy(74). Consequently, in previous experiments reported independently by at least three laboratories, autophagy was found to result in reduced levels of p62(33, 46, 71). Depleted levels of p62 in turn would be expected to allow Keap1 to bind the Nrf2 transcription factor and lead to increased degradation of Nrf2 protein.

As expected, I did find that p62 levels decrease in breast cancer cells during glucose deprivation. Surprisingly, however, I found that Nrf2 protein levels and Nrf2 activity are actually *increased* in the setting of glucose deprivation, which is an unexpected finding in light of previously reported findings that decreases in p62 lead to decreases in Nrf2. These increases in Nrf2 protein levels and activity were not dependent on autophagy, since Nrf2 increased even in cells with knock-out of the ATG7 or Beclin1 autophagy genes. Furthermore, even though the Nrf2 pathway can be activated by oxidative stress (77), I found no evidence that increases in levels of Nrf2 are a response to increased levels of reactive oxygen species (ROS) during glucose deprivation. Rather, I found that Nrf2 levels increase in breast cancer cells during glucose deprivation, even when cultures that were treated with the antioxidant, N-acetyl cysteine. As summarized in the figure below, our experimental data shows that decreases in p62 *do not* lead to decreased levels of Nrf2. Rather, levels of Nrf2 are increased. Even though levels of ROS are also increased, and increased levels of ROS are capable of increasing Nrf2, our data suggests that these increases in ROS *are not* driving the increases in Nrf2. Therefore,

we believe that some other mechanism is involved in elevating levels of Nrf2 during glucose deprivation.

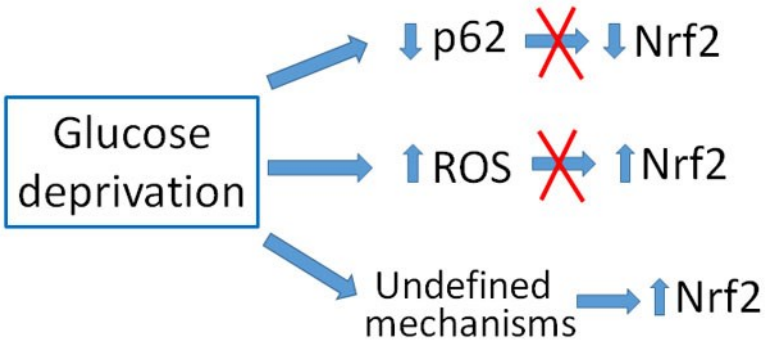

Figure 7.1 Schematic of the effect of glucose deprivation on Nrf2 signaling

An important question to address in future studies, is how is Nrf2 activated by glucose deprivation? Previous experiments have shown that Nrf2 pathway can be activated by various transcriptional, epigenetic, and post-translational mechanisms(96).

Rather than focusing on mechanisms that cause increased Nrf2 activity during glucose deprivation, I directed additional studies to investigate what role the Nrf2 pathway might play in cell survival during glucose deprivation. Comparing cell survival in MCF7 cells that had been transfected with shRNA targeting KEAP1 (to increase Nrf2) or targeting Nrf2 (to decrease levels of this transcript), I found that loss of Nrf2 led to markedly decreased survival in glucose-deprived cultures. Thus, in addition to autophagy, induction of Nrf2 is critical for cell survival in the setting of glucose deprivation. Knock-down of Keap1 had minimal effects on survival in these experiments, likely because levels of Nrf2 were already elevated in glucose-deprived cells, and knock-down of Keap1 had minimal additional protective effect.

Nrf2 is a transcription factor known to activate multiple enzymes with antioxidant properties, including NADPH dehydrogenase quinone 1 (NQO1). We found that increased Nrf2 levels correlated with increased levels of NQO1, indicating that Nrf2 activity, as well as the level of Nrf2 protein, is increased during glucose deprivation. To explore how glucose deprivation and Nrf2 activity affect cellular levels of ROS, we used 2',7'-dichlorodihydrofluorescein diacetate (DCFDA), a cell-permeable, chemically reduced form of fluorescein, as an indicator for ROS in cells and found that cells with knockdown of Nrf2 showed time-dependent increases in ROS when cultured in glucose-free medium. By contrast, cells with knockdown of Keap1 showed significantly less ROS when cultured in glucose-free medium. These results show that cellular levels of ROS are increased during glucose deprivation, and that Nrf2 is important for attenuating this oxidative stress.

Since these data point to Nrf2 activity playing a critical role for cell survival during nutrient deprivation-induced autophagy, I then tested whether the antioxidants, N-acetyl cysteine and glutathione, could also protect cell survival during autophagy. Indeed, these two antioxidants protect survival of Nrf2-depleted cells experiencing glucose deprivation, suggesting that the role of increased Nrf2 during glucose deprivation involves coordinating an anti-oxidant response. However, N-acetyl cysteine does not affect the decreases in p62 or the increases in Nrf2 levels during glucose deprivation, suggesting that neither of these processes are initiated by high levels of reactive oxygen species.

In addition to studying the role of Nrf2 in the survival of cancer cells during glucose deprivation, I briefly looked at survival in hypoxia. Nrf2 was shown to be

important in survival of hypoxic cells although to a lesser extent than in glucose deprivation. Studying the effect of combining hypoxia and glucose deprivation resulted in the finding that both the autophagic and antioxidant pathways were decreased. HIF1α expression was also attenuated in the hypoxia/glucose deprivation combination growth condition which may result from decreases in ATP levels according to some studies (52-54).

Finally in experiments unrelated to Nrf2, the effect of glucose deprivation on breast cancer receptors targeted by drugs was investigated. Breast cancer can be divided into 3 main categories based on receptor status with 2 of those subtypes- ER/PR positive and HER2 positive- having therapies targeted toward the estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2). Both tamoxifen and trastuzumab bind to ER and HER2 respectively and block signaling resulting in decreased growth. Initial response to the targeted therapy eventually turns to resistance. Therefore, I wanted to study how glucose deprivation, autophagy, and receptor downregulation might be involved in the drug resistance. I found that ER and HER2 are downregulated in glucose deprivation possibly through a transcriptional mechanism. Hypoxia alone had no effect on the receptors, however in combination with glucose deprivation, the receptors remained decreased. Both autophagy and proteasomal degradation of the receptors proved not to be the likely mechanism for downregulation as neither affected the levels of receptors when inhibited. With the finding that receptors were downregulated, it was interesting to see that the targeted drug therapies were still efficacious during glucose deprivation. In my studies, they were even better at cell killing than in complete media.

This is very paradoxical as the target receptor is present in the complete media cells and therefore the drug has its place to act on. In glucose free media the receptor is no longer present and thus the drug should not have a mode of action. Therefore it would be interesting to study the mechanism of action during glucose deprivation. In addition to tamoxifen having an unexpected effect, trastuzumab was found to be efficacious in estrogen receptor positive cell lines despite its use in the treatment of HER2 positive cancers.

In summary, glucose deprivation is a situation commonly encountered by cancer cells, particularly in tumors with disordered tissue architecture and areas of cellular ischemia. Glucose deprivation is not necessarily lethal to these cells because of protective mechanisms, which we have investigated due to their potential importance in understanding barriers to successful cancer treatment. I identified two major mechanisms of cell survival in the setting of glucose deprivation: autophagy and induction of Nrf2. Interestingly, these two survival mechanisms appear to be activated independently of one another, and in fact, the activation of the Nrf2 pathway that we observed is contrary to expectations, based on previous studies that have investigated connections between autophagy and Nrf2. Nrf2 was also found to be important in hypoxia. In unrelated studies to Nrf2, both estrogen and human epidermal growth factor receptors were discovered to be downregulated in response to glucose deprivation perhaps through a translational mechanism. Despite this downregulation of ER in MCF7 and T47D cells, 4-hydroxytamoxifen was still efficacious. Major questions that should be addressed in future studies include, a) what are molecular mechanisms of Nrf2 activation during glucose deprivation?, b) can the cell survival mechanisms activated by glucose

deprivation be targeted for improving cancer therapy?, c) to what extent are these survival mechanisms more critical for survival of cancerous cells in tumors than cells in normal tissues?, and finally in relation to targeted therapies, d) what drives the downregulation of receptors in breast cancer, why are targeted therapies still effective in this setting and how can this be exploited in human treatment?

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96. Bryan HK, Olayanju A, Goldring CE, Park BK. The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation. Biochem Pharmacol. 2013 Mar 15;85(6):705-17.

Curriculum Vitae

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Ph.D

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EDUCATION

JOHNS HOPKINS UNIVERSITY, SCHOOL OF MEDICINE, BALTIMORE, MD

Ph.D., Pathobiology, July 2016

Thesis Dissertation: "Survival Pathways in Breast Cancer Cells During Glucose Deprivation"

Mentor: Dr. Edward Gabrielson, M.D.

UNIVERSITY OF MARYLAND BALTIMORE COUNTY (UMBC), BALTIMORE, MD

Bachelor of Science in Biochemistry and Molecular Biology, May 2009

Minor in Biology and Psychology, Cum Laude.

RESEARCH EXPERIENCE

Department of Pathology, 2009-2016

Research Focus: The role of autophagy and the Nrf2 pathway in breast cancer cells during glucose deprivation and how these

pathways might be exploited to further enhance cancer treatment.

Techniques: mammalian cell culture, immunoblotting, clonogenic assays, immunofluorescence, measuring reactive oxygen species, lentivirus transfection, reverse transcriptase-PCR

U.S. ARMY RESEARCH LABORATORY, ADELPHI, MD

Optics Branch, Bio-Inspired Sensors and Devices Team, Summers 2005-2008, January 2006-2007

Research Focus: Cellulolytic bacteria and microbial fuel cells.

Techniques: polymerase chain reaction (PCR) assays, real time PCR, UV/Vis spectroscopy, gel electrophoresis, fluorescent in situ hybridization, anaerobe chamber, nanodrop spectrophotometer, preparation of growth media and bacterial cultures, growth studies, bacterial plasmid isolation and insertion into microbes, bacterial cell DNA isolation and amplification.

COMMITTEES AND LEADERSHIP ROLES

President, Graduate Student Association, July 2014 –July 2015

Mentor, High school Students' Senior Project, May 2014

Vice President of Finance, Graduate Student Association, July 2013 –June 2014

Parking Representative, Graduate Student Association, Aug 2012 –August 2016

Representative, Graduate Student Association July 2011-July 2013

AWARDS

Second Place in the 2016 JHGCC Biotech & Healthcare Case Competition

Second Place in the 2015 JHGCC Mini-Case Competition

Won Best Local Medical Product at the "A Call for Innovation" competition during JHU Entrepreneurship Week, May 2014.

First Place in the Pathobiology Annual Retreat Poster Competition, Sept 2014.

PRESENTATIONS AND PUBLICATIONS

A.L. Walker, A. Singh, J. Woo, B. Poore, A. Le, and E. Gabrielson, "Survival Pathways in Breast Cancer Cells During Glucose Deprivation" (In Preparation) 2016

Alyssa Walker, "Nrf2 Determines Survival in Cancer Cells Undergoing Autophagy due to Nutrient Deprivation", Johns Hopkins Pathobiology Graduate Program Retreat, Sept 2015

Alyssa Walker, "Autophagy and Breast Cancer Drug Resistance", Johns Hopkins Medical Institution Pathology Grand Rounds, July 2011

A.L. Walker and C.W. Walker, Jr. Biological Fuel Cell and an Application as a Reserve Power Source. J. Power Sources 160 (2006) 123-129.

C.W. Walker, Jr. and **A.L. Walker**. Biological Fuel Cell Functional as an Active or Reserve
Power Source. U.S. Army R&D Technical Report ARL-TR-3840, June 2006.